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DIFFERENTIAL INTRACELLULAR OXIDATION AND REDUCTION OF INDICATORS IN RELATION TO FISSION

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IN EARLIER studies of intracellular reduction of methylene blue and Janus green it was found that in zoöid chains of the rhabdocoel, *Stenostomum*, the rate of reduction decreased posteriorly from the head regions of zoöids. Also in *Tubifex* and several species of earthworms, all forms which do not undergo fission, two reduction gradients opposed in direction—one with rate of reduction decreasing from the head posteriorly for a certain distance, the other with rate decreasing anteriorly from the segment-forming region immediately anterior to the anal segment—were found to be present (Child, 1934; Child and Rulon, 1936). Segment-formation in these oligochetes continued at least through a considerable part of postembryonic life. However, in some oligochetes segment-formation does not continue after formation of a certain number of segments. In such forms the posterior segment-forming gradient may be expected to disappear when the full number of segments has differentiated. In fully developed *Aeolosoma* individuals, which consist of a small number of segments, and in one earthworm species in which segment-formation had evidently ceased, only a single reduction gradient with decrease in rate from the anterior end was found in the earlier studies. Evidence was also presented showing that in *Aeolosoma*, *Nais*, and *Stylaria*—all forms which undergo fission with morphological differentiation of zoöids preceding separation—alteration of the gradient pattern in a definite manner is associated with the appearance of each new zoöid; each zoöid represents a new reduction-gradient pattern.

At intervals from 1943 on, further study of indicator gradient patterns, particularly in their relation to fission, has been undertaken as material became available. Patterns of primary oxidation, i.e., intracellular oxidation of dyes entering the organism in completely reduced form without preceding staining, of intracellular reduction of oxidized dyes, and reoxidation following reduction and also of intracellular indophenol reaction and reduction have been determined, and their modifications under certain experimental conditions have been investigated.

MATERIAL AND METHODS

The rhabdocoel turbellarian, *Stenostomum*, is in certain respects very favorable material for indicator studies. The formation of chains consisting of zoöids

in various developmental stages, resulting from continuous increases in length of each zoöid, and successive formations of new zoöids from each posterior region as certain lengths are attained provides a wide range of material in a single chain of several zoöids. At least two species of *Stenostomum* have been used, one, apparently *S. grande*, originally described from the Chicago region. Under cultural conditions permitting rapid growth, chains up to 8 zoöids, occasionally even more, may occur in this species, with a total length of 3–4 mm. More frequently, however, chains consist of 3–5 zoöids, and these served as the chief material. A much smaller species, apparently *S. leucops*, usually forming chains of only 2–3 zoöids in the cultures and little more than 1 mm. in length, also served as material. Figures 1 and 2 are diagrammatic outlines of 3-zoöid chains of the two forms. Both developed in large numbers in a culture medium consisting of fragments of yeast cake in water with the addition of a little yeast as appeared necessary. A culture was sometimes satisfactory for 2–3 weeks or even longer, but new cultures could be started at any time.

Aeolosoma with the characteristics of *A. hemprichii* was used extensively as material. This primitive oligochete formed chains in the yeast culture medium usually consisting of 3 or 4 zoöids; occasionally 5-zoöid chains were found. Both *Stenostomum* and *Aeolosoma* were originally obtained from *Elodea* growing in tanks on the Stanford University campus. Several hundred individuals or chains of each of these forms were used with the indicators. Two zoöid chains of the oligochete, *Chaetogaster*, were also obtained. This form has been found only rarely in the region about Palo Alto and Stanford.

In the South American oligochete, *Nais paraguayensis*, separation into a variable number of pieces, "fragmentation," occurs without morphologically visible fission planes or any other evidence of zoöid-formation. Anterior and posterior regeneration of the pieces occurs rapidly. It was thought that indicator patterns of this species might be of interest in throwing light on the question whether particular body-levels of separation might be physiologically, even though not morphologically, predetermined. This material was obtained through the kindness of Dr. Willis Johnson from his laboratory cultures. The dyes used as indicators of oxidation and reduction were chiefly methylene blue and Janus green (diazine green). Nile blue sulphate and neutral red were also used in some cases for comparison. Intracellular indophenol formation and reduction served for comparison with the dyes. Concentrations of methylene blue used ranged from 1/400,000 to 1/20,000; of Janus green from 1/200,000 to 1/50,000.

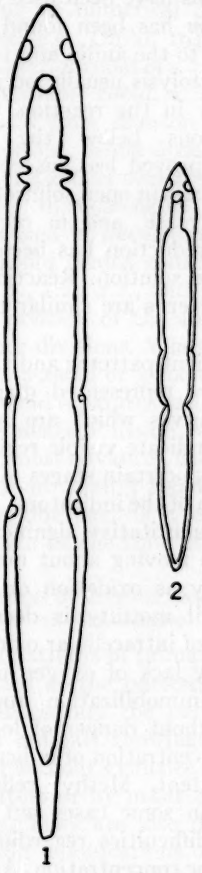
For primary intracellular oxidation, i.e., oxidation in completely reduced solution or on aeration following a period in reduced dye, the animals were usually sealed with exclusion of air in glass cells containing 0.5 ml. of dye solution reduced by a minute amount of sodium hydrosulphite ($Na_2H_2S_2O_4$). This is not appreciably toxic even in much higher concentrations than those used for dye reduction, and it reduces the dye at once; but, with long periods in the reduced solutions or in water with hydrosulphite, effects of lack of oxygen appear. For short exposures to reduced methylene blue 1–2 ml. of reduced dye solution, on large depression slides with the animals, may be left open to the air. Without agitation the deeper levels of such solutions, several millimeters from the surface, remain reduced for 10 minutes or

more. This procedure makes it possible to observe what happens as motor activity of the animals carries them into regions of higher or lower oxygen content. If they move to the surface or margin, intracellular oxidation may be almost instantaneous, often with differential toxic effect or even cytolysis in a few seconds in the higher dye concentrations. When some excess of hydrosulphite is present, reduction may occur on return of intact animals to regions of low oxygen content. Primary intracellular oxidation occurs in sealed or open preparations of the completely reduced colorless form of Janus green.

For intracellular reduction, material is stained by oxidized dye or material which has undergone primary oxidation in reduced dye is used. Hydrosulphite has been used in most cases to decrease the oxygen content of the dye solution or water containing the animals, and both sealed and open preparations have served for reduction. Reduction can, of course, be brought about more slowly by oxygen decrease resulting from the oxygen uptake of animals sealed in small volumes of solution or of water with exclusion of air.

In primary intracellular oxidation of methylene blue and in reoxidation following reduction, the blue color of the oxidized dye appears; in reduction the color disappears completely. With Janus green, oxidation usually consists in the appearance of the partially oxidized red stage. Complete intracellular oxidation to blue-green sometimes occurs with high oxygen content of the solutions. In intracellular reduction of completely oxidized Janus green, there is change of color from blue-green to red, the color of the partially reduced dyë. Further reduction toward or to colorless occurs only in extremely low oxygen and is soon followed by cytolysis unless oxygen content of the solution is increased without delay. Other details of procedures concerned with intracellular oxidation and reduction of dyes are described in later sections of the paper as occasion requires.

The indophenol reaction, i.e., the intracellular formation of the deep-blue indophenol from para-aminodimethylaniline (dimethyl-para-phenylenediamine) and α-naphthol, believed to be catalyzed by an oxidase, or, more specifically, cytochrome oxidase, has also been used as far as possible in the study



FIGS. 1 and 2.—Chains consisting of 3 zoöids of the two species of *Stenostomum* serving as material. Fig. 1, *S. grande* (?). Fig. 2, *S. leucops* (?).

of patterns and particularly for comparison with the patterns brought to light by the dyes. As described in earlier papers (e.g., Child, 1944*a*, *b*), stock solutions of the reagents made daily or oftener consisted of 1 small drop (40 drops = 1 ml.) of liquid anilin, slightly warmed if necessary to liquefy, in 10 ml. of water and 1 mg. or less of naphthol, also in 10 ml. of water. For the reaction 1 small drop of each reagent in 1 ml. of water has been regarded for convenience as the standard concentration, and concentrations above or below this have been used as desired. *Stenostomum* has been found so highly susceptible to the anilin and to indophenol that cytolysis usually occurred relatively early in the reaction, even with concentrations below the standard. *Aeolosoma* proved less susceptible. The reaction occurs in open solutions without oxygen decrease, and in certain cases secondary reduction has been observed in the same solution. Reaction and reduction patterns are similar to the dye patterns.

The gradient patterns and their modifications are represented diagrammatically by curves which are merely attempts to indicate visible regional color differences at certain stages of oxidation or reduction of the indicators. They have no exact quantitative significance. The animals are moving about more or less continuously as oxidation or reduction occurs, until motility is decreased by toxic effect of intracellular oxidized indicators or by lack of oxygen in reduced solutions. Immobilization appears impossible without danger of local differences in concentration of indicators or of oxygen content. Methyl cellulose has been used in some cases but does not avoid the difficulties regarding oxygen and indicator concentration. Anesthetics may alter the patterns. In the curves representing the patterns, the head of the individual or chain and, of course, also the head regions of developing zoöids are at or toward the left side of the figure. In the curves of differential oxidation, greater distance above the base line indicates a higher rate of oxidation. In the reduction curves, less distance above the base line indicates a higher rate of reduction. In an earlier paper, concerned with reduction pattern only, greater distance above the base line indicated more rapid reduction, i.e., the reduction curve of that paper were essentially like the oxidation curves of the present paper (Child and Rulon, 1936). More recently, a single curve was used for both oxidation and reduction in a planarian (Child, 1948*a*). However, in order to indicate certain modifications of gradient patterns appearing in different zoöids under certain conditions, separate curves for oxidation and reduction are regarded as necessary in this paper. In describing the patterns, the terms "posteropetal" and "anteropetal" are used as in earlier papers, meaning progression of color change toward more posterior or more anterior regions.

Stenostomum

FORMATION OF ZOÖIDS

Under natural and good cultural conditions, localization and development of zoöids and, consequently, of fission planes occur in a definite order. With increase in length of the individual, the head region of a new zoöid is determined at a certain distance from the primary head, and a fission plane is established immediately anterior to the new head. The length of body from the primary head to the head of the new zoöid varies very little under fairly uniform conditions but can be altered experimentally. After the establishment of the first fission plane, each zoöid increases in length and development of the head of the second

zoöid progresses. Rate of development of the head and of the fission plane and rate of increase in length differ widely under different conditions. With relatively little food and slow increase in length, separation may occur in the 2-zoöid stage. Development of the head and the fission plane are less retarded by lack of food than is growth in length. With better nutritive conditions, the anterior zoöid, which increases in length more rapidly than the posterior zoöid, divides again, giving rise to 3-zoöid forms like Figures 1 and 2. With still more abundant food, other zoöids may be formed before separation occurs at the most advanced fission plane, and 4-, 5-, 7-, and 8-zoöid chains, occasionally chains with a still larger number of zoöids, result. At certain stages in the development of chains an odd number of zoöids is distinguishable because zoöids at the more anterior levels of the chain increase in length more rapidly, and consequently new zoöids appear in their posterior regions earlier than in zoöids at more posterior levels. In other words, there is a gradient decreasing posteropetally in rate of growth in length. Also the length which a zoöid attains before a new zoöid is determined in its posterior region depends on rate of growth in length in relation to rate of development of its head. In other words, the range of dominance of the head region of a zoöid increases with progress of development of its head. Each new head region is characterized by development of new cephalic ganglia, and these must determine more or less reorganization of the nervous system progressing posteriorly from the new head. Determination and development of a new zoöid head can occur only in a region more or less physiologically isolated from a more anterior head already developed or in process of development. If growth in length has been rapid in relation to rate of head development and reorganization of the nervous system, as is the case with abundant food, the new zoöid head is determined at a shorter distance posterior to a head in process of development than when growth in length is slow (Child, 1941, pp. 27, 303–4 and literature cited there). These relations between head development and length of zoöid preceding determination of another zoöid from its posterior region are directly visible in *Stenostomum* chains of 3 or more zoöids.

According to numerical designations used earlier (e.g., Child, 1941, pp. 399–405), the anterior zoöid of a 2-zoöid chain is 1, the posterior zoöid, 2. The 3-zoöid chain results from division of zoöid 1 into 1.1. and 1.2. before division of zoöid 2. Chains of 4 zoöids consist of 1.1., 1.2., 2.1., and 2.2. In such chains, zoöid 1.2. is more advanced in development than is 2.2. Five-zoöid chains consist of zoöids 1.1.1., 1.1.2., 1.2., 2.1., and 2.2. and result from division of the anterior zoöid before other divisions. When separation occurs in a 2-zoöid or 3-zoöid chain, the posterior zoöid separates as an individual without evidence of fission. With separation at the most advanced fission plane in chains of more than 3 zoöids, the separated posterior part is a 2-zoöid or a 3-zoöid chain at the time of separation.

PATTERNS OF PRIMARY OXIDATION IN *Stenostomum*

Certain patterns of primary oxidation are regarded as the patterns of normal animals. These are the patterns which appear in animals showing normal activity and without evidence of injury by the indicators or by other experimental conditions. They are also distinguished from certain modifications of the oxidation pattern appearing with insufficient oxygen, excess of hydrosulphite, or other experimental conditions. These normal

patterns are, in general, oxidation gradients decreasing in rate posteropetally from the head region of the individual and of each zoöid of a chain; but it must be noted that in the earliest stages of zoöids the gradient is sometimes not distinguishable with entire certainty; also a slight increase in rate of oxidation appears in the extreme posterior regions of certain zoöids. At all body-levels a decrease in rate from ventral to dorsal regions appears. These are patterns of the body-wall. A slight posteropetal decrease in rate is usually evident in the entoderm.

Figures 3, 4, 5, and 6 each indicate an early and a later stage of primary oxidation in an individual, a 2-zoöid, a 3-zoöid, and a 5-zoöid chain. At the early stage, only the head of the individual (Fig. 3) or the two most advanced heads have begun to show color (Figs. 4–6). At the later stage the gradient pattern appears in all zoöids. These patterns appear with a wide range of concentrations of methylene blue reduced to colorless by hydrosulphite, but some aeration after a period in the reduced dye may be necessary for complete oxidation. With Janus green reduced to colorless, oxidation to the red diethyl safranin shows the same patterns. The pattern of the indophenol reaction is also the same, but *Stenostomum* is rather highly susceptible to injury by the anilin and by indophenol, and it cytolyzes before intracellular concentration of indophenol has become high.

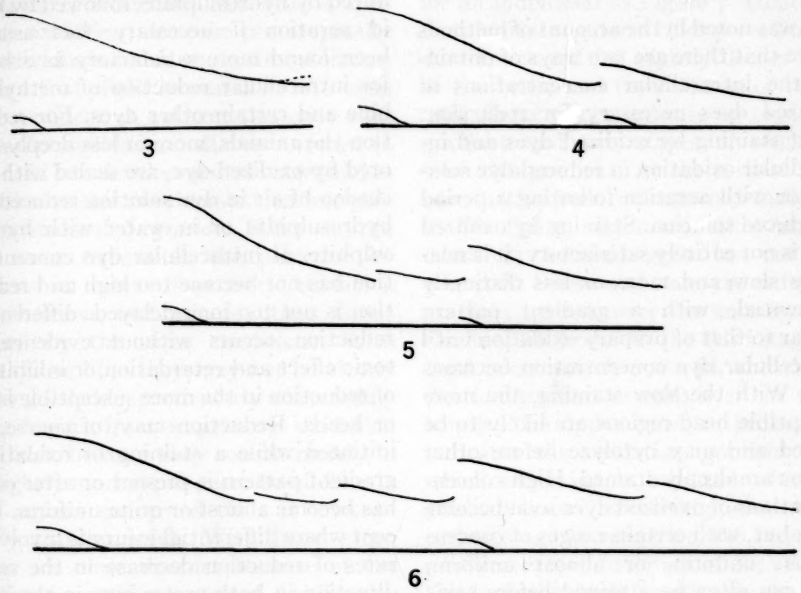
The fully developed head is highly motile, and, as the figures indicate, oxidation in it is distinctly more rapid than elsewhere. The sensory ciliated pits oxidize more rapidly than do other parts of the head. From the fully developed head, the rate of intracellular oxidation decreases more steeply than from the heads of zoöids. In individuals of *S. grande* in which the tail is still developing, there is a slight increase in rate of oxidation posteriorly, indicated in Figure 3 by the short, broken line at the right end of the curve. Apparently, the functional activity of the fully developed tail of *S. grande* may, at least in some cases, determine a slightly higher rate of oxidation there than in the adjoining region. *Stenostomum leucops* lacks an elongated tail, and, after the injury and stimulation resulting from separation have disappeared, no distinct increase in rate of oxidation at the posterior end has been observed. This condition is indicated by the unbroken line at the right end of the curve in Figure 3.

In zoöids with posterior ends immediately anterior to heads of zoöids in advanced developmental stages, a slight increase in rate of primary oxidation usually appears posteriorly. This is indicated at the posterior end of the anterior zoöid in the 2-zoöid chain of Figure 4 at the later stage of oxidation. In Figure 5 it appears at the posterior end of the early middle zoöid, which is anterior to the advanced head of the posterior zoöid but is not evident in the posterior region of the anterior zoöid, probably because the changes in condition associated with the fission plane and the head region of the early, middle zoöid are still slight. In Figure 6 the posterior increase in rate appears in the second, third, and fourth zoöids from the anterior end but not in the posterior region of the anterior zoöid, which in this chain, as in Figure 5, adjoins a very early head region. It is suggested that one or more of three conditions or factors may be concerned in these posterior increases in rate of primary oxidation. These are: increase in oxidase activity involved in development of the fission plane; anterior spread of the activity of the developing head immediately posterior to the region concerned; the beginning of posterior regeneration in the zoöid. The posterior ends of the

anterior zoöids of Figures 5 and 6 adjoin very early fission planes and heads; this may account for the failure to observe a posterior increase in rate in these zoöids.

With hydrosulphite in considerable excess of the amount necessary to reduce the dyes, intracellular oxidation may be only partial, with more or less complete reversal of the gradient pattern, i.e., oxidation may progress anteriorly in zoöids, except for the extreme posterior ends, and anterior regions of the more advanced zoöids may fail to oxidize until the solution is aerated. Following aeration, the normal gradient pattern may appear. With excess of hydrosulphite in methylene blue solutions, there may be little or no intracellular oxidation, except perhaps in the entoderm, until the solution is aerated. With rapid aeration, the normal pattern appears; with slow and slight aeration, the gradients may be reversed. With Janus green, oxidation to red occurs at lower oxygen concentration than oxidation of methylene blue; consequently, a greater excess of hydrosulphite is required for reversal of the gradients or prevention of oxidation. With very low concentrations of reduced methylene blue, the entoderm may oxidize while the body-wall reduces the dye and remains entirely without color until the solution is aerated.

With sufficient available oxygen, intracellular oxidation may continue in the reduced dye solution or on aeration following a period in the reduced solution, until color is almost or quite uniform over the entire length of the individual or chain. With rapid aeration following a few minutes in reduced solution, uniform color may be attained almost at once.



FIGS. 3–6.—Stages of the gradient patterns of primary intracellular oxidation in reduced dye solutions and of the indophenol reaction in *Stenostomum*. In these and all following figures the head of the individual or the anterior zoöid of the chain is at the left. Fig. 3, an individual. Fig. 4, a 2-zoöid chain. Fig. 5, a 3-zoöid chain. Fig. 6, a 5-zoöid chain.

Except for noting that the gradient patterns of primary oxidation of dyes and of the indophenol reaction suggest gradient patterns of oxygen uptake, discussion of their significance is postponed to the final section of the paper.

DIFFERENTIAL REDUCTION AND REOXIDATION IN *Stenostomum*

It was noted in the account of methods above that there are two ways of obtaining the intracellular concentrations of oxidized dyes necessary for reduction: direct staining by oxidized dyes and intracellular oxidation in reduced dye solution or with aeration following a period in reduced solution. Staining by oxidized dyes is not entirely satisfactory; it is relatively slow and more or less distinctly differential, with a gradient pattern similar to that of primary oxidation until intracellular dye concentration becomes high. With the slow staining, the more susceptible head regions are likely to be injured and may cytolyze before other regions are deeply stained. High concencentrations of oxidized dyes soon become toxic; but, with certain ranges of concentration, uniform, or almost uniform, color can often be attained before toxic effect and retardation of reduction are evident. Oxidized Janus green has been found more satisfactory than methylene blue and various other dyes for direct staining. The brilliant red color of the partially reduced dye makes the gradient patterns distinct, even with very slight staining, and there is apparently less differential staining than with methylene blue. So far as it has been possible to determine by direct visual comparison, the same depth of intracellular color appears to be less toxic when attained by rapid staining with higher, than by slow staining with lower, dye concentrations, at least within a certain range of concentrations. This difference has also been observed in various other organisms. Apparently, after the oxidized dye enters the cell, there is a gradual adsorption or accumulation on or in, or reaction with, certain cell constituents which become visible as stained granules or bodies, and, as this occurs, the dye becomes toxic.

The other method—a period in dye reduced by hydrosulphite, followed by rapid aeration if necessary—has usually been found more satisfactory as a basis for intracellular reduction of methylene blue and certain other dyes. For reduction the animals, more or less deeply colored by oxidized dye, are sealed with exclusion of air in dye solution reduced by hydrosulphite or in water with hydrosulphite. If intracellular dye concentration has not become too high and reduction is not too long delayed, differential reduction occurs without evidence of toxic effect and retardation or inhibition of reduction in the more susceptible head or heads. Reduction may, of course, be initiated while a staining or oxidation-gradient pattern is present or after color has become almost or quite uniform. Except where differential injury is involved, rates of reduction decrease in the same direction in both cases; but, in the progressive transformation of a primary oxidation pattern or a pattern of direct staining into a reduction pattern, certain color differentials appear which are not present when depth of color preceding reduction is almost or quite uniform. Primary oxidation of completely reduced Janus green preceding reduction is less useful than oxidation of methylene blue because, under the usual conditions, intracellular oxidation is only partial (to the red diethyl safranin) and reduction from this to colorless, while it does show the gradient patterns, is so soon followed by differential cytolysis that its usefulness is limited. With rapid and extreme aeration, oxidation toward or to the com-

pletely oxidized blue-green dye can sometimes be obtained, and differential reduction of this to red is possible, though the pattern may be modified by differential toxic effect of the oxidized dye. Direct staining by oxidized Janus green has been more commonly used as a basis for reduction with Janus green.

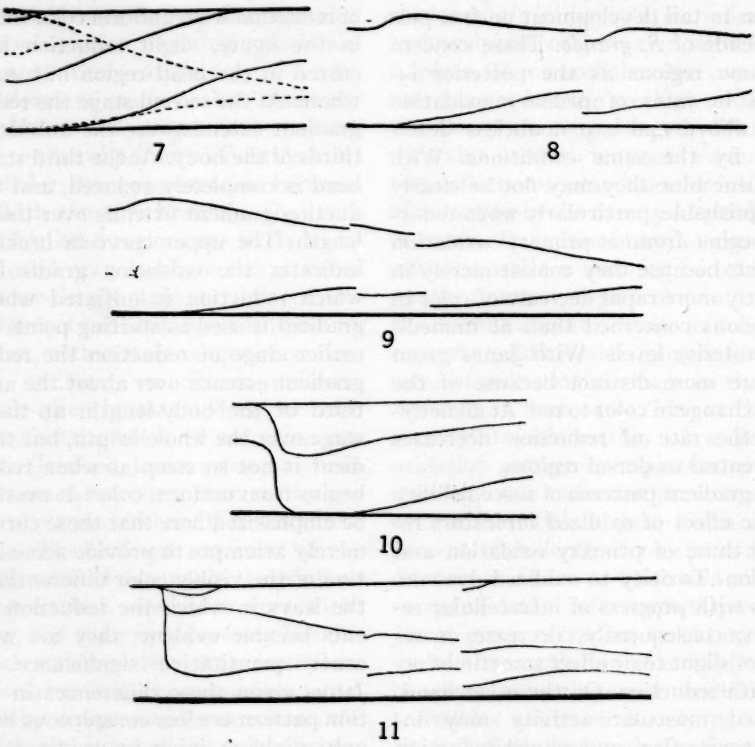
Rates of reduction, like rates of primary oxidation, decrease posteropetally in individuals and in the zoöids of chains, except that slight increases in rate seem to be evident at extreme posterior ends of earlier zoöids immediately anterior to heads in late developmental stages and in relation to tail development on free posterior ends of *S. grande*. These concern the same regions as the posterior increases in rates of primary oxidation noted above and are doubtless determined by the same conditions. With methylene blue they may not be clearly distinguishable, particularly when reduction begins from a primary oxidation gradient, because they consist merely in a slightly more rapid decrease of color in the regions concerned than at immediately anterior levels. With Janus green they are more distinct because of the earlier change in color to red. At all body-levels the rate of reduction decreases from ventral to dorsal regions.

The gradient patterns of susceptibility to toxic effect of oxidized indicators resemble those of primary oxidation and reduction. Toxicity to oxidized dyes decreases with progress of intracellular reduction; consequently, decrease or reversal of slight toxic effect sometimes occurs with reduction. On the other hand, increased muscular activity may increase toxic effect and retard reduction differentially. This often results from the intense irritation on exposure to strong light in oxidized methylene blue. Repeated trial with a wide range of dye concentrations is necessary for certainty in avoiding differential injury and in determining when it occurs.

In order to call attention to the chief differences between reduction from an oxidation gradient and that from virtually uniform coloration by oxidized dye, with methylene blue as indicator, both reduction patterns are represented for an individual in Figure 7. Unbroken lines indicate uniform color preceding reduction, except for slightly deeper color of the head, and three stages of reduction. Broken lines indicate an oxidation gradient and two stages of reduction from this gradient. At the earliest stage of reduction from uniform color indicated in the figure, slight reduction has occurred in the head region but not elsewhere. At the second stage the reduction gradient extends over the anterior two-thirds of the body. At the third stage the head is completely reduced, and the reduction gradient extends over the whole length. The upper curve in broken line indicates the oxidation gradient from which reduction is initiated when the gradient is used as starting point. At the earlier stage of reduction the reduction gradient extends over about the anterior third of the body-length, at the later stage over the whole length, but the gradient is not so steep as when reduction begins from uniform color. It must again be emphasized here that these curves are merely attempts to provide some indication of the visible color differentials and the ways in which the reduction gradients become evident; they are without exact quantitative significance. With Janus green these differences in reduction pattern are less conspicuous because only slight staining by oxidized dye is necessary as a basis for reduction and the staining gradient is slight. In reduction from red to colorless, the gradient patterns are similar to those of reduction to red.

In the chains the relations of the reduction patterns to fission are essentially the same in reduction from uniform color and from an oxidation gradient or a staining gradient. An early and a later stage of reduction in a 2-zoöid chain are represented in Figure 8. Reduction began from a slight Janus green staining gradient. At the earlier stage indicated in the upper curve, reduction has begun in the two head regions, with somewhat higher rate in the fully developed head of the anterior member than in that of the posterior zoöid. At the later stage, reduction is complete anteriorly in the anterior member, less advanced in the head of the posterior zoöid, and the reduction gradient extents over the entire length of both.

In Figure 9 reduction in a 3-zoöid chain from a rather late stage of a methylene blue primary oxidation gradient or a slight staining gradient is indicated. The earlier stage (upper curve) shows reduction in the head regions of the anterior and posterior zoöids and very slight reduction anteriorly in the middle, very early zoöid. In the earliest visible



FIGS. 7–11.—Stages of reduction patterns and some of their modifications in *Stenostomum*. Fig. 7, individual reduction patterns; unbroken lines indicate stages of reduction from uniform color, except slightly deeper head region (*upper line*); broken lines, stages of reduction from an oxidation gradient; further explanation in text. Fig. 8, two stages of reduction in a 2-zoöid chain. Fig. 9, two stages of reduction in a 3-zoöid chain. Fig. 10, modification of reduction pattern in an individual by retardation of reduction in the fully developed head as a result of toxic effect of oxidized indicators. Fig. 11, modification of reduction pattern with complete inhibition of reduction in the fully developed head of a 3-zoöid chain.

stages of this zoöid and others in later fissions, it is often uncertain whether reduction has occurred anteriorly or not. At the later stage (lower curve) rate of reduction decreases posteropetally in all three zoöids, except for slight increases in rate, not indicated in Figure 9, in the extreme posterior region of the middle zoöid and at the posterior end of the posterior zoöid in certain stages. These posterior increases in rate correspond to those observed in primary oxidation patterns, and possible conditions or factors concerned were noted above in connection with oxidation patterns. Reduction may occur in fully developed heads and in heads of the more advanced zoöids in oxidized methylene blue, except in high concentrations.

Alterations of reduction pattern result from differential injury by oxidized dyes and decrease or loss of reducing capacity in the injured regions. The fully developed preoral head region is most susceptible to such injury. Figures 10 and 11 serve as examples. In Figure 10 two stages of reduction from uniform depth of color are represented in an individual with retardation in the head region. At the earlier stage there is no appreciable reduction in the head, but rate of reduction is highest at a level slightly posterior to the head and decreases posteropetally. At the later stage (lower curve) some reduction has occurred in the head, but it is still much deeper in color than the rest of the animal. Rate of reduction decreases posteropetally elsewhere in the body.

In Figure 11 reduction from uniform color and from an oxidation gradient with complete absence of reduction in the fully developed head is represented for a 3-zoöid chain. At the early stage of reduction from uniform color, represented by the upper curve, reduction is beginning just posterior to the anterior head and less rapidly in the head region of the posterior zoöid. The middle curve represents an early stage of reduction from an oxidation gradient with reduction beginning just posterior to the anterior head, in the head region of the posterior zoöids, and probably in the extreme anterior region of the middle zoöid. The later stage (lower curve) shows the rate of reduction decreasing posteropetally in all three zoöids, except in the fully developed head. The head region of the posterior zoöid reduces more rapidly than the most rapidly reducing postcephalic region of the anterior zoöid. In the later stages there is no great difference in gradient pattern with reduction from uniform color and from an oxidation gradient. Some difference in steepness of the gradients may be visible.

In some advanced 3-zoöid chains and in chains of more than 3 zoöids the head posterior to the most advanced fission plane is almost completely developed, and separation may occur at any time. In these chains this head is more susceptible to the toxic effect of the oxidized dyes than earlier stages are, and reduction may be retarded in it. The gradient pattern of injury by oxidized dyes is like that of oxidation and reduction; injury progresses posteropetally, with decreasing effect in retarding or inhibiting reduction. Consequently, with certain degrees of injury, reversal in direction of the reduction gradient may occur over more or less of the zoöid length, i.e., retardation of reduction increases anteropetally. This occurs more frequently in the anterior zoöid than elsewhere.

Following staining by primary oxidation of low concentrations of reduced methylene blue or by similar concentrations of the oxidized dye, secondary reduction often occurs, even in oxidized solution. These reductions show the usual gradient patterns but may occur

only in the anterior regions of individuals or zoöids and may not be complete in those regions. In oxidized methylene blue, 1/100,000 or lower concentrations, reversal of staining gradients may occur in greater or less degree. The more anterior regions of individuals or zoöids reduce, or otherwise decolorize, the dye as rapidly as it enters. The body-wall of the more anterior regions may remain completely unstained, while the entoderm becomes deeply stained. Similar reversals or absence of staining often occur in higher concentrations of oxidized methylene blue if animals are allowed to aggregate in considerable numbers and so decrease the oxygen locally in their environment.

Intracellular reoxidation of reduced methylene blue and various other dyes, brought about by rapid and adequate increase of oxygen content of the reduced solution or by removal of the animals to well-aerated water, shows the same gradient patterns as primary oxidation (Figs. 3–6). However, with extremely rapid oxygen increase, the color may become uniformly deep so rapidly that the gradient pattern is visible only for a few seconds. On the other hand, with slow and slight oxygen increase, the oxidation gradients may be partially or completely reversed in direction. If intracellular concentration of oxidized dye does not become too high, intracellular reduction and reoxidation of methylene blue can often be repeated two or three times before the reduction pattern is altered by differential toxic effect. In fact, repetition of reduction and reoxidation is useful as a means of determining when modifications of pattern by differential toxic effect appear. The use of open solutions of reduced methylene blue, with oxidation occurring slowly at the surface and animals moving into regions of higher or lower oxygen content, with consequent oxidation or reduction, was described in the earlier section concerned with methods.

Janus green is less useful than methylene blue and other dyes for reoxidation. Differential reoxidation toward, or to, blue-green may occur in some cases, usually with rapid and great increase of oxygen. Also animals in red, partially reduced solution may stain blue-green. Differential reduction from red to colorless is followed so soon by cytolysis that reoxidation to red is usually possible only by immediate rapid oxygen increase following the reduction. However, this reoxidation is chiefly useful for demonstrating that it is possible in some cases. It has been found of no great value for showing reoxidation patterns of entire individuals or chains because the regions which reduce to colorless most rapidly, cytolyze before reduction occurs at all levels.

Experimental conditions which reverse the longitudinal reduction gradient may also obliterate or reverse the ventro-dorsal gradient. Even in the head this reversal has often been observed with methylene blue, the dorsal side becoming partially or completely reduced, while the ventral side still shows color. Both fully developed and developing heads may show this reversal.

Aeolosoma

FORMATION OF ZOÖIDS AND SEGMENTS

The sequence of fissions in *Aeolosoma* is the same as in *Stenostomum*. When the individual attains a certain length, a new head region and fission plane are localized at a certain distance from the original head. The anterior member of the 2-zoöid chain grows in length more rapidly and divides earlier than the posterior member. Zoöid lengths attained before localization of a new head differ in different zoöids, as in *Stenostomum*. In the cultures

separation usually occurred at the most advanced fission plane before more than 3 zoöids were distinguishable. Chains of 4 or 5 zoöids were occasionally seen but usually separated at the most advanced fission plane on slight stimulation.

In various oligochetes in which formation of new segments continues for a considerable time, or perhaps indefinitely in postembryonic life, two gradients are present, as indicated by differential susceptibility to KCN (Hyman, 1916), and by differential dye reduction (Child and Rulon, 1936). One of these, which may be regarded as the primary polar gradient, decreases posteropetally from the anterior end; the other, the secondary gradient of segment-formation and development, decreases anteropetally from the segment-forming region immediately anterior to the anal segment. Undoubtedly, there are specific differences in the physiological activities characteristic of the two gradients, but both show similar quantitative susceptibility and oxidation-reduction differentials.

The fully developed *Aeolosoma* indivual consists of a small number of segments. Formation of segments does not continue indefinitely. Each new zoöid originating from the posterior region of a pre-existing individual or zoöid includes the region in which new segments have recently developed or are still developing; and, after it is determined as a new zoöid, segment-formation continues or begins again in its posterior region. The number of distinguishable segments in a zoöid at the beginning of its development differs according to its position in the chain. The very short zoöid resulting from the division of the anterior member of a 2-zoöid chain consists of few distinguishable segments. The zoöid resulting from division of the posterior member of a chain is longer and includes more segments at the time of its formation. Segment-development in the posterior regions of zoöids determines more or less distinct oxidation-reduction gradients, opposed in direction to the primary gradients and corresponding to the gradients of segment-formation in other oligochetes.

Aeolosoma is, in general, less susceptible than *Stenostomum* to injury in connection with the indophenol reaction; consequently, this reaction and intracellular reduction of indophenol are more useful for comparison with the dyes than in *Stenostomum*. So far as observed, the indophenol patterns and dye patterns are identical as regards directions of the gradients.

THE HEAD OF *Aeolosoma*

Certain characteristics of the head region are of interest in relation to indicator patterns. In dorsal or ventral view the prostomium is rounded in outline; dorsiventrally it is flattened. It is highly motile, and, obviously, its sensory functions are highly important to the animal. Primary oxidation of dyes and the indophenol reaction are much more rapid in the fully developed prostomium and the peristomium than elsewhere and more rapid ventrally than dorsally, and the head is much more susceptible to injury by the oxidized indicators with decrease or loss of capacity to reduce. As compared with other parts, this susceptibility seems to be relatively greater than in *Stenostomum*. As injury occurs, motility of the head decreases and ceases, the prostomium loses its flattened form and may finally cytolyze, while other regions are intact and active. As in *Stenostomum*, developing heads of zoöids, at least after the earliest stages, oxidize and, until injured, reduce indicators more rapidly than do postcephalic levels of the same zoöids but are less susceptible to injury by intracellular oxidized indicators than

are fully developed and much more active heads.

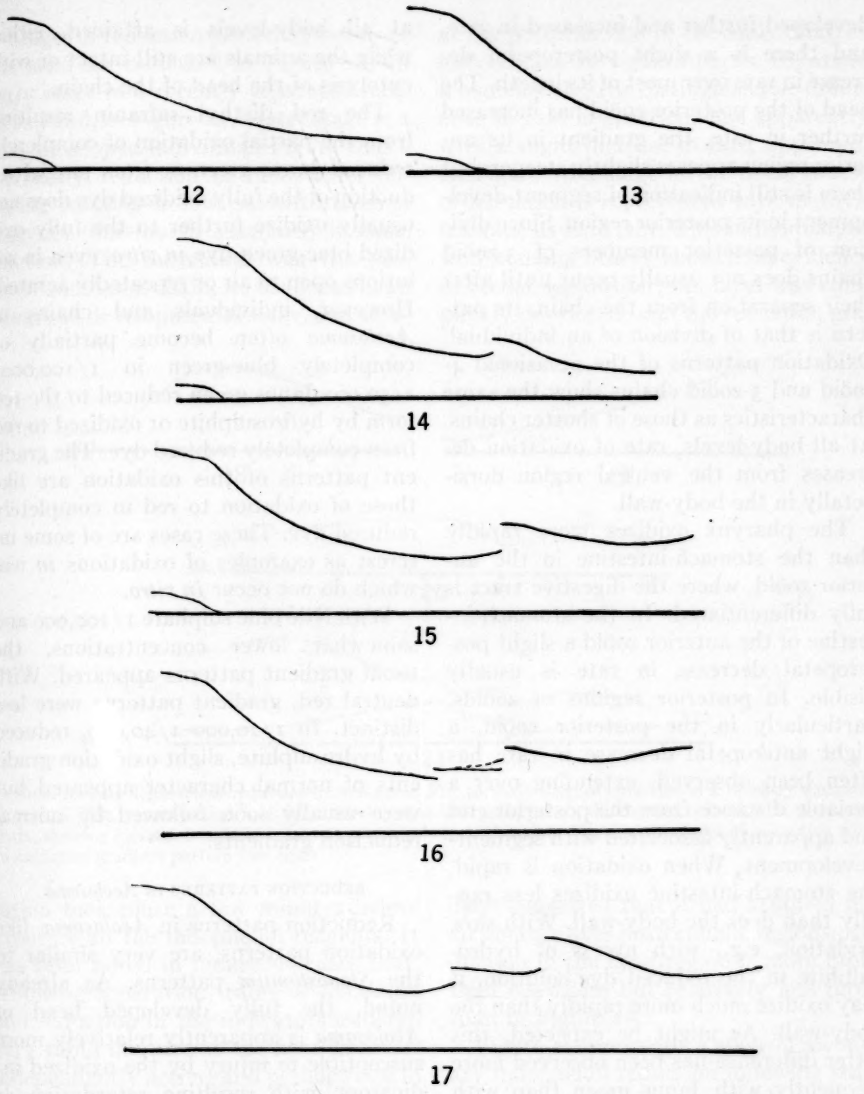
PRIMARY OXIDATION PATTERNS IN *Aeolosoma*

In its general relations to fission, the longitudinal gradient pattern of primary oxidation of indicators does not differ greatly from that of *Stenostomum*. The decrease in rate from the fully developed head seems to be somewhat steeper than in the rhabdocoel, as is indicated in Figure 12 for an individual and in Figures 13–17 for the anterior members of chains. In the longer undivided animals, i.e., those which have apparently attained complete development as regards number of segments, the rate of the indophenol reaction and of primary oxidation of dyes decreases posteropetally over the whole length (Fig. 12). In somewhat shorter individuals an increase in rate may appear in the posterior regions. In the light of further evidence to be presented, this difference is believed to indicate that segments are still developing posteriorly in the shorter individual and that this development is completed in the longer individual.

In the early stages of development of a second zoöid from the posterior region of the individual, there is a slight increase in rate in the new head region but usually no marked change in the gradient at more posterior levels or in the anterior member (Fig. 13). At a slightly later stage (Fig. 14) the head of the posterior zoöid shows further increase in rate of oxidation, and a slight increase has sometimes been observed at the extreme posterior end of the anterior member, as indicated in Figure 14. As in *Stenostomum*, this may indicate merely increased activity in connection with the developing fission plane or an anterior spread of the activity of the developing head of the second zoöid; or it may indicate a very early stage of posterior regeneration and segment-formation in this region of the anterior member. At a still later stage of the 2-zoöid chain the head region of the second zoöid has increased further in rate and a distinct increase in rate often appears in the posterior region of both zoöids. This increase is more distinct and extensive than are the posterior increases in rate in *Stenostomum* and is believed to be associated with segment development (Fig. 15). In the posterior zoöid the rate of oxidation sometimes decreases anteropetally over most of the zoöid length.

In its earliest distinguishable stages the third zoöid in order of appearance (zoöid 1.2.), formed by division of the anterior member of the 2-zoöid chain, is only one-fifth to one-sixth the length of the remaining anterior zoöid (Fig. 16). It represents approximately the region supposedly involved in segment-formation preceding the division. Its head region usually shows a very slightly higher rate of oxidation than does the new posterior end of the anterior zoöid (Fig. 16). There may be no distinguishable oxidation gradient in this zoöid in its early stages or a slight posteropetal increase in rate, as indicated by the unbroken and the broken line in Figure 16. These conditions in the middle zoöid are believed to result from segment-development, which began in this region before it became a new zoöid. There is no distinguishable change in the new posterior region of the anterior zoöid; segment-development has apparently not begun again since the division but is apparently continuing in the posterior zoöid.

At the later 3-zoöid stage of Figure 17 a slight increase in rate of oxidation appears in the extreme posterior region of the anterior zoöid, as in the 2-zoöid chain of Figure 14. The following zoöid may also show a very slight increase in rate at its extreme posterior end. Its head has



FIGS. 12–17.—Stages of gradient patterns of primary intracellular oxidation in *Aeolosoma*. Fig. 12, primary oxidation in a fully developed individual, early stage below with oxidation only in head region; later stage above with oxidation gradient over whole length. Fig. 13, early and later stage of primary oxidation in an early 2-zoöid chain. Fig. 14, early and later stage of primary oxidation in a 2-zoöid chain slightly more advanced than Fig. 13, showing slight increase in rate posteriorly in anterior zoöid. Fig. 15, primary oxidation in a still more advanced 2-zoöid chain with distinct increase in rate posteriorly in both zoöids. Fig. 16, oxidation pattern in early 3-zoöid chain; the short middle zoöid may show little or no gradient (*unbroken line*) or a slight increase in rate posteriorly (*broken line*), and there is increase in rate posteriorly in the posterior zoöid (see text). Fig. 17, oxidation in a more advanced 3-zoöid chain with slight increase in rate posteriorly in the anterior zoöid and the middle zoöid and two opposed gradients in the posterior zoöid.

developed further and increased in rate, and there is a slight posteropetal decrease in rate over most of its length. The head of the posterior zoöid has increased further in rate, the gradient in its anterior region appears slightly steeper, but there is still indication of segment-development in its posterior region. Since division of posterior members of 3-zoöid chains does not usually occur until after their separation from the chain, its pattern is that of division of an individual. Oxidation patterns of the occasional 4-zoöid and 5-zoöid chains show the same characteristics as those of shorter chains. At all body-levels, rate of oxidation decreases from the ventral region dorsipetally in the body-wall.

The pharynx oxidizes more rapidly than the stomach-intestine in the anterior zoöid, where the digestive tract is fully differentiated. In the stomach-intestine of the anterior zoöid a slight posteropetal decrease in rate is usually visible. In posterior regions of zoöids, particularly in the posterior zoöid, a slight anteropetal decrease in rate has often been observed, extending over a variable distance from the posterior end and apparently associated with segment-development. When oxidation is rapid, the stomach-intestine oxidizes less rapidly than does the body-wall. With slow oxidation, e.g., with excess of hydrosulphite in the reduced dye solution, it may oxidize much more rapidly than the body-wall. As might be expected, this latter difference has been observed more frequently with Janus green than with methylene blue.

Figures 12–17 indicate stages in the course of primary intracellular oxidation in reduced dye solution or stages of the indophenol reaction. However, if intracellular dye oxidation or the indophenol reaction is permitted to continue, uniform, or almost uniform, depth of color at all body-levels is attained, either while the animals are still intact or with cytolysis of the head of the chain.

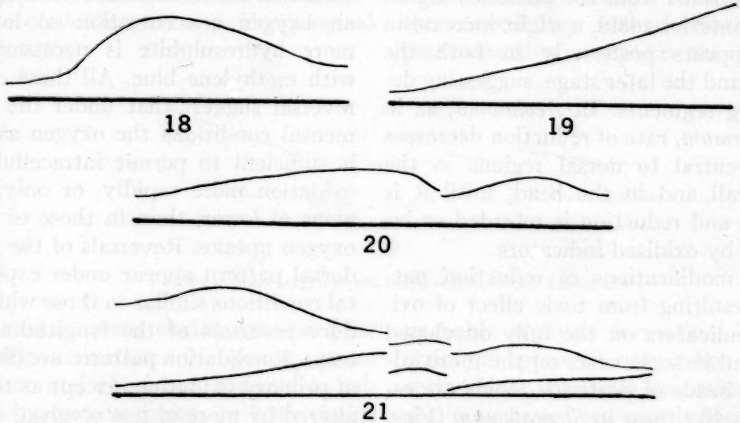
The red diethyl safranin resulting from the partial oxidation of completely reduced Janus green or from partial reduction of the fully oxidized dye does not usually oxidize further to the fully oxidized blue-green dye *in vitro*, even in solutions open to air or repeatedly aerated. However, individuals and chains of *Aeolosoma* often become partially or completely blue-green in 1/100,000–1/50,000 Janus green reduced to the red form by hydrosulphite or oxidized to red from completely reduced dye. The gradient patterns of this oxidation are like those of oxidation to red in completely reduced dye. These cases are of some interest as examples of oxidations *in vivo* which do not occur *in vitro*.

With Nile blue sulphate 1/200,000 and somewhat lower concentrations, the usual gradient patterns appeared. With neutral red, gradient patterns were less distinct. In 1/20,000–1/40,000, reduced by hydrosulphite, slight oxidation gradients of normal character appeared but were usually soon followed by normal reduction gradients.

REDUCTION PATTERNS IN *Aeolosoma*

Reduction patterns in *Aeolosoma*, like oxidation patterns, are very similar to the *Stenostomum* patterns. As already noted, the fully developed head of *Aeolosoma* is apparently relatively more susceptible to injury by the oxidized indicators, with resulting retardation or inhibition of reduction. Also, increases in rate of reduction in posterior regions, apparently associated with development of new segments, are greater than the very slight increases noted in *Stenostomum*. Otherwise, no important differences in the reduction patterns and their modifications have been observed.

Stages of reduction preceding injury of the head are indicated in Figure 18, an early stage in a recently separated individual and, in Figure 19, a later stage in a fully developed individual. Figure 18 is a secondary reduction of indophenol following reaction in low concentrations of reagents (one-fourth standard) in open solution. The individual was the posterior zoöid of a chain, its head was in an advanced developmental stage, and separation took place a few minutes before exposure to the indophenol reagents. It has been noted in numerous cases that, following separation, rates of oxidation and reduction of the indicators undergo very rapid increase as the head becomes independently active, and perhaps also in part on account of the wound resulting from separation, although this is slight in the advanced head. The head in Figure 18 reduces much more rapidly than other regions and becomes almost colorless, while slight indophenol reaction with the usual posteropetal gradient is present over most of the body. There may be a few not yet fully differentiated segments at the posterior end of the individual, as indicated at the right of Figure 18, where a region with little distinguishable difference in rate of reaction, but apparently with a slight increase in rate, appeared. In Figure 19 a stage in the usual course of reduction is represented with methylene blue as indicator. This individual, as well as many others, showed no evidence of recent separation; the head was completely developed, and the reduction gradient suggested that it had attained full dominance over postcephalic regions. Reduction in the head region is more rapid than elsewhere but relatively less rapid than in Figure 18.



FIGS. 18–21.—Differential reduction in *Aeolosoma*. Fig. 18, early stage of reduction in a recently separated individual. Fig. 19, reduction in a fully developed individual. Fig. 20, differential reduction in a 2-zoöid chain, showing increase in rate of reduction posteriorly in both zoöids. Fig. 21, two stages of reduction from an oxidation gradient pattern (see text).

Figure 20 represents a stage of secondary indophenol reduction following reaction with one-fourth standard reagents, as it has been repeatedly observed in 2-zoöid chains. The rate of reduction decreases posteropetally from the two head regions, but there is an increase in rate in the posterior region of each zoöid, probably indicating developing segments.

In Figure 21 two stages of methylene

blue reduction from an oxidation-gradient pattern are represented. At the earlier stage (above) the three head regions have reduced more rapidly than adjoining postcephalic regions, but reduction has not progressed far posteriorly. At the later stage the reduction gradient is present over the entire length of anterior and posterior zoöids, with no evidence of segment development posteriorly. In the short, middle zoöid, recently formed from the posterior region of the anterior zoöid, a slight increase in rate appears posteriorly in both the earlier and the later stage, suggesting developing segments. In *Aeolosoma*, as in *Stenostomum*, rate of reduction decreases from ventral to dorsal regions in the body-wall and in the head, until it is injured and reduction is retarded or inhibited by oxidized indicators.

The modifications of reduction patterns resulting from toxic effect of oxidized indicators on the fully developed head and in some cases on the more advanced heads of posterior zoöids are essentially like those in *Stenostomum* (Figs. 10 and 11). With progress of injury, decreasing posteropetally from the head, particularly in the anterior zoöid, which is longer than others, more or less reversal in direction of the reduction gradient may occur. In oxidized methylene blue, 1/100,000 and lower concentrations, reduced by hydrosulphite, partial or complete reversal of the staining gradient in the longer zoöids has usually been observed. The more anterior levels and, in some cases, regions of probable segment-development reduce or otherwise decolorize the dye as rapidly as it enters. In methylene blue, reduced with slight excess of hydrosulphite, reversal of the primary oxidation gradient by differential reduction may be obtained. With slow or slight increase of oxygen following exposure to methylene blue, reduced with a greater excess of hydrosulphite, reversal of oxidation gradients may also occur; but, if rapid oxygen increase follows, the normal oxidation pattern may appear. With sufficiently low concentrations of indophenol reagents, reversal of the reaction-gradient pattern appears, or the reaction may be only partial. With sufficient excess of hydrosulphite, reversal of the primary oxidation pattern of Janus green can be obtained; but intracellular oxidation from colorless to red occurs at an oxygen concentration so low that more hydrosulphite is necessary than with methylene blue. All these cases of reversal suggest that under the experimental conditions the oxygen available is sufficient to permit intracellular dye oxidation more rapidly, or only, in regions of lower, than in those of higher, oxygen uptake. Reversals of the ventro-dorsal pattern appear under experimental conditions similar to those which produce reversals of the longitudinal patterns. Reoxidation patterns are like those of primary oxidation, except as they are altered by more or less reversal.

In neutral red 1/20,000 and 1/40,000 reduced by hydrosulphite, slight oxidation gradients were usually soon followed by secondary reduction with normal gradient patterns. With Nile blue sulphate 1/200,000–1/400,000, reduction patterns and their modifications were similar to those with methylene blue. Higher concentrations were rapidly toxic.

Chaetogaster

Chaetogaster was rarely found in the tanks of the Stanford School of Biological Sciences and was never seen in material collected elsewhere in the region about Palo Alto. Indicator patterns were obtained from only two chains, one with a single advanced fission plane and perhaps very early stages of two other zoöids in the posterior regions of the anterior and

posterior members, the other distinctly a stage of a 3-zoöid chain with a possible very early stage of another zoöid. It was known from earlier observations on fission in *Chaetogaster* material frequently collected in the Chicago region that the sequence of fissions and the differences in relative lengths of zoöids in relation to position in the chain and developmental stage are much like these characteristics in *Stenostomum* and *Aeolosoma* and evidently result from essentially similar relations of dominance and physiological isolation.

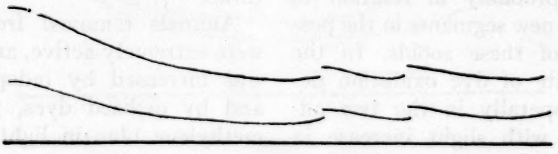


FIG. 22.—Two stages of primary oxidation in *Chaetogaster*

The *Chaetogaster* chains were used for primary oxidation in Janus green 1/50,000 and 1/100,000, reduced to colorless by hydrosulphite. In both chains oxidation-gradient patterns like those of *Aeolosoma* appeared in each zoöid. Two stages of the oxidation pattern of the 3-zoöid chain are represented in Figure 22. At the earlier stage indicated by the lower curve in the figure, the rate of oxidation decreases posteropetally from the head regions in all three zoöids, but with a slight increase in rate in each posterior region, probably associated with formation of new segments, as in *Aeolosoma*. At all body-levels the gradients became visible first in the ventral body-wall, and rate of oxidation decreased dorsipetally. The ventrodorsal gradient appeared much steeper, i.e., the difference in rate of oxidation from ventral to dorsal region seemed to be much greater, than in *Stenostomum* and *Aeolosoma*. During 20 minutes in the reduced dye, with further progress of oxidation, the gradients were still visible, and constriction increased or appeared at each fission plane. A constriction also appeared in the posterior region of the anterior zoöid at approximately the level at which the head of a new zoöid would appear, suggesting that another zoöid might perhaps be physiologically determined, but no fission plane could be distinguished. A very slight increase in rate of dye oxidation seemed to be present at the constriction, with decrease in rate posteropetally, except in the region of probable segment formation. The constriction appeared only when toxic effects of the dye were becoming evident and may have been merely local muscular contraction; but its appearance in the region where the head of a new zoöid would be localized is at least suggestive. Because of its appearance only at a late stage of oxidation and its uncertain character, it is not represented in Figure 22.

The transparency of the body-wall of *Chaetogaster* made it possible to observe oxidation in the segmental ganglia of the ventral nerve cord over much of the length of the anterior zoöid, where they were not obscured by coelomic cells and other tissues. Intracellular oxidation of the dye appeared to be more rapid in the ganglia than in other internal organs, and rate of oxidation decreased posteriorly from segment to segment. Unfortunately, other parts of the central nervous system were less clearly visible, and it could not

be determined whether they showed new gradients corresponding to the zoöids.

The muscular pharynx oxidized about as rapidly as the body-wall at that body-level and apparently with a slight posteropetal decrease in rate, the "oesophagus" less rapidly, with slight posteropetal decrease. The stomach-intestine of the anterior zoöid was partly hidden by other tissues but showed slow and slight oxidation without a clearly distinguishable gradient. In the posterior zoöid of the chain and the middle zoöid, rate of intestinal oxidation appeared to decrease anteropetally, probably in relation to development of new segments in the posterior regions of these zoöids. In the other chain, rate of dye oxidation decreased posteropetally in the two advanced zoöids, with slight increase in rate in the posterior region of each. These data on *Chaetogaster*, though, of course, in need of confirmation on much more material, are believed to be of sufficient comparative interest to justify their inclusion in this paper.

Nais paraguayensis

Individuals of this species may attain a length of 30–35 mm. but undergo fragmentation. In an experimental study of the conditions determining fragmentation, Hyman (1938) has found that animals above a certain length will usually separate into several pieces within 24 hours when removed from the boiled-lettuce cultures to pure water without food at 25°–30° C. Animals 10 mm. or less in length do not fragment, and the number of pieces increases with increase in length but is not always the same for a given length. Lengths of pièces can be altered by culturing at different temperatures and at different pH. Although there is no visible indication of fission planes, Hyman concludes that the body-levels at which separation occurs are predetermined in mature animals, but apparently in relation to cultural and other conditions.

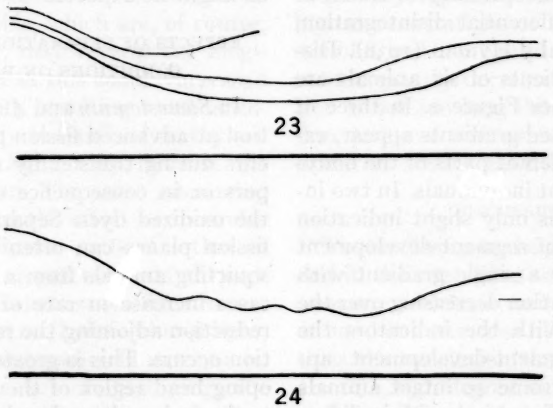
In the study of indicator patterns, indophenol reagents, in concentrations from one-half standard to twice standard (see methods), oxidized Janus green 1/50,000 and 1/60,000, and oxidized methylene blue 1/20,000–1/100,000 were used with reduction of the dyes by hydrosulphite and by respiration of a number of animals sealed in a small volume of solution, and reoxidation by aeration after reduction, often repeated several times.

Animals removed from the culture were extremely active, and their activity was increased by indophenol reagents and by oxdized dyes, particularly by methylene blue in light on the microscope stage. Motor activity often became so extreme that partial anesthesia by chloretone was used in some cases in order that gradient patterns might be more clearly seen and also to decrease possible effects of the intense activity on pattern. Intact animals, 25–35 mm. in length, without evidence of recent regeneration; regenerating pieces of various stages, resulting from fragmentation; and pieces isolated by section at various body-levels, also in different stages of regeneration, served as material.

As in other oligochetes, regenerating or recently regenerated parts are readily distinguishable from old parts, since they are more nearly transparent, and the levels of junction of old and new parts are distinct, even in later stages of regeneration. In most cases several individuals or pieces were used in a single lot because they were less active than those isolated singly. Exact numbers in each lot were not always recorded, but the total number in which oxidation-reduction patterns were observed was more than 150. In intact animals without evidence of re-

cent regeneration two opposed longitudinal gradients in rate of indophenol reaction and of reduction and reoxidation of dyes appeared in the body-wall—one with decrease in rate from the prostomium posteropetally over a variable number of segments, the other the gradient of segment-development, with decrease in rate anteropetally from the posterior region, usually in those individuals with completed regeneration, from a level immediately anterior to the anal segment and also extending over a variable number of segments. In the longer animals these two gradients did not usually extend over the entire body-length but were separated by a region varying somewhat in position and length, in which no definite longitudinal gradient was visible. In the shorter individuals this region without gradient was short or virtually absent. These oxidation-gradient patterns in three individuals, two long, one short, are represented in Figure 23 as examples of the types of patterns observed in many individuals. In some of the longer animals one, two, or three local regions of slightly more rapid oxidation appeared (Fig. 24). These were most conspicuous in the indophenol reaction. They apparently had no relation to levels of separation and may have indicated merely local contractions.

Rates of reduction also decrease posteropetally from the prostomium and anteropetally from the posterior region, with the anal segment often distinguishable by its slightly lower rate than the region of segment-formation immediately anterior to it. The anal segment requires special mention. In posterior regeneration of pieces a new anal segment develops, but the region of continued segment-formation is immediately anterior to it. In earlier stages of regeneration, the anal segment oxidizes and reduces the indicators as rapidly as does the region of segment-formation anterior to it; but it gradually undergoes differentiation, and its rate decreases below that of the segment-forming region anterior to it (Fig. 24). This difference apparently persists as long as new segments continue to be produced. Similar conditions were noted earlier in *Tubifex* (Child and Rulon, 1936). At all body-levels, rates of



FIGS. 23 and 24.—Differential primary oxidation in *Nais paraguayensis*. Fig. 23, the usual longitudinal gradient pattern in long and short individuals. Fig. 24, oxidation with zones of apparently more rapid oxidation in middle region, perhaps merely regions of contraction, giving appearance of more rapid oxidation

oxidation and reduction decrease dorsipetally from the ventral body-wall.

Regeneration, both anteriorly and posteriorly, is extremely rapid following either fragmentation or section at different levels. As in various other oligochetes, regeneration anteriorly is limited to prostomium and a small number of segments, while posterior regeneration continues until maximum length is attained or perhaps indefinitely with decreasing rate.

Differential susceptibility to KCN, as indicated by differential disintegration, was determined by Hyman (1938). Disintegration gradients of six animals are represented in her Figure 2. In three of these, two opposed gradients appear, extending over different parts of the body-length in different individuals. In two individuals there is only slight indication of the gradient of segment-development and in one only a single gradient with rate of disintegration decreasing over the entire length. With the indicators the gradient of segment-development appeared in all of some 40 intact animals examined but differed in length in different individuals. These differences between disintegration and oxidation-reduction patterns may mean only that fragmentation, with resulting regeneration with segment-development, was more frequent in the culture from which the indicator material was obtained.

In early stages of regeneration, when anterior and posterior new tissue were only 0.2–0.4 mm. in length, the indophenol reaction and dye reduction and reoxidation were more rapid in it than in the old parts until toxic effects occurred, but it was also more susceptible to injury and retardation or inhibition of reduction, and it cytolyzed earlier than the old tissues. Reversal of the reduction gradient in the regenerating parts, both anterior and posterior, occurred in consequence of differential injury by oxidized indicators. In early stages of regeneration, reversal was often complete in the new tissue with methylene blue. In later stages it was more commonly partial, involving only the prostomium or prostomium and one or two segments posterior to it at the anterior end and posteriorly only a posterior region of the anteropetal gradient. Use of chloretone for partial anesthesia seemed in some cases to decrease gradient differentials, as might be expected.

EFFECTS OF SEPARATION AND OTHER CONDITIONS ON PATTERN

In *Stenostomum* and *Aeolosoma* separation at advanced fission planes often occurs during transfer by means of droppers or in consequence of irritation by the oxidized dyes. Separation at earlier fission planes can often be induced by squirting animals from a dropper. In all cases increase in rate of oxidation and reduction adjoining the region of separation occurs. This is greater in the developing head region of the zoöid posterior to the fission plane than in the region anterior to the plane, which is the posterior region of a zoöid. The wound resulting from separation at earlier fission planes is less extensive than that following section at other levels and closes more rapidly. With separation at advanced planes, the wound is relatively slight and soon closes; but in both cases the isolation of the head region posterior to the plane is followed by distinctly more rapid oxidation and reduction of indicators. Increases in rate also occur anteriorly and posteriorly to section at other levels than fission planes, and the increase is greater posterior than anterior to the level of section, but apparently less than in the head region posterior to a fission plane following separation. Whether such differences appear anterior and posterior to levels of

separation in *N. paraguayensis* was not determined, but there can be little doubt that they occur. Similar effects anterior and posterior to levels of separation by section have been observed in the hydroid, *Corymorpha palma* (Child and Watanabe, 1935; Watanabe, 1935; Child, 1941, pp. 101–2), in hydra and a planarian (Child, 1948*a*), and in amphibian embryos and larvae (Child, 1948*b*).

Strong contractile activity increases the rate of oxidation-reduction. Contractile activity at the more posterior levels of posterior zoöids, which are, of course, free posteriorly, may reverse the longitudinal gradient in this zoöid. Increased muscular activity resulting from irritation, particularly in oxidized methylene blue in strong light, may increase the rate of reduction. The most interesting case observed of the effect of muscular activity on indicator pattern is that of the disgorging of gut contents through the mouth in *Stenostomum*. This occurs often in oxidized methylene blue in light, evidently in consequence of extreme irritation. In the act of disgorging, the mouth opens to a diameter greater than that of the head or body with the mouth closed, the head is altered in shape, and there is less extreme contraction at adjoining postcephalic levels. It has been repeatedly observed that, in animals rather deeply stained but still moving normally up to the moment of disgorging, the entire head and sometimes adjoining postcephalic levels become colorless at once and in some cases disintegrate completely within a few seconds.

It does not follow from these effects of muscular activity that this activity alone determines normal gradient patterns. The patterns appear independently of differential muscular contractions, but the muscular activity of the fully developed head of the individual or the anterior zoöid in *Stenostomum*, *Aeolosoma*, and *Chaetogaster* and regions immediately posterior to it doubtless plays a part in determining the steepness of the gradient in this individual or zoöid. The earlier stages of zoöids may contract when the whole chain contracts, but their head regions are not independently motile, and independent muscular contraction has not been observed in other parts of their bodies. Nevertheless, the gradient patterns appear and become increasingly steep without independent motility. With very rapid and deep oxidation in high concentrations of reduced methylene blue and dim light, muscular activity is soon greatly decreased; but, if reduction is not too long delayed, the reduction patterns and even patterns of reoxidation are not essentially altered.

DISCUSSION

The similarity in the gradient patterns and in their relations to fission in *Stenostomum*, *Aeolosoma*, and, as the few data at hand indicate, also in *Chaetogaster* justifies the conclusion that these patterns are to a high degree independent of the specific differences in constitution of these forms. The apparently greater difference between the head, particularly the prostomium and peristomium, and adjoining postcephalic regions in *Aeolosoma* and *Chaetogaster* than in *Stenostomum*, which seems to be evident in rates of oxidation and reduction of indicators and in susceptibility to injury by them, suggests a somewhat higher degree of cephalization in the oligochetes than in *Stenostomum*.

It is of interest that the gradient patterns of differential disintegration in KCN observed in *Aeolosoma* by Hyman (1916) show gradients of decrease in rate of disintegration in the same directions as the oxidation-reduction gradients in intact animals. The disintegration gradients, plotted body-level against time, are

steeper than the gradients represented in the figures of this paper, but this difference has no physiological significance. The disintegration gradients indicate the progress of disintegration along the body; the oxidation-reduction gradients described in this paper represent stages in progress of oxidation or reduction.

The presence of ventrodorsal oxidation and reduction gradients in the head and at all body-levels in the rhabdocoel, *Stenostomum*, and the three oligochetes is also of interest. A similar ventrodorsal gradient is present in the planarian body (Child, 1948*a*) and in certain other rhabdocoels and insect embryos so far as examined (Child, unpublished). This gradient and the localization ventrally of the nerve cord are apparently correlated. The evidence at hand concerning gradient patterns in cephalized bilateral invertebrates indicates that the central nervous system is localized at or near the highest levels of the gradient pattern. In the planarian the cephalic ganglia develop in the high region of the polar gradient of early developmental stages, and they, as well as the nerve cords, are nearer the ventral than the dorsal surface of the body (Child, 1941, Fig. 64, *A*, p. 179). In annelids and, according to the evidence from certain insect embryos, also in arthropods the cephalic ganglia develop at or near the high end of the polar gradient, and the nerve cord is localized in the high region of the ventrodorsal gradient. However, the position of the so-called "brain" near the dorsal side of the head in annelids and arthropods appears to conflict with these suggestions. Some other factor—perhaps the relation of the cephalic nervous system to the anterior region of the alimentary tract—seems to be concerned. In the vertebrate the cephalic nervous system develops in the higher levels of the primary polar gradient and of the dorsiventral gradient; at all postcephalic levels the dorsal region is the high region of the dorsiventral gradient, and from this the postcephalic nervous system develops. Teleosts and amphibians are excellent examples of these relations (Child, 1943*a*, *b*, 1945*b*, 1948*b*).

The fully developed head of the individual or chain shows more rapid indophenol reaction and oxidizes and, until injured, also reduces more rapidly than do developing heads of other zoöids, except perhaps in some cases the heads of advanced posterior zoöids almost ready to separate. However, as pointed out above, it is more susceptible to injury by oxidized indicators than other parts are, with resulting retardation or inhibition of reduction. Its great muscular activity and probably also its sensory differentiation are believed to be factors in its oxidation-reduction characteristics. Muscular activity increases rate of oxidation and reduction of indicators, and regions of sensory differentiation generally show higher rates of oxidation and reduction and are more susceptible to injury than general ectoderm. The margins and lateral lobes of the planarian head are cases in point (Child, 1948*a*). The ciliated pits and the extreme tip of the head of *Stenostomum* show the indophenol reaction and oxidize reduced dyes more rapidly than do other parts of the head region. Reduction in the ciliated pits has not been observed, but the tip of the head reduces before other parts and also cytolyzes earlier. The much higher rate of oxidation and reduction and the greater susceptibility to injury by oxidized dyes in the prostomium of *Aeolosoma*, and particularly its ventral side, have been noted above.

Each new zoöid originates from the posterior region of an individual or a preexisting zoöid and does not at once lose the characteristics of a posterior region.

Its development represents the gradual imposition of a new gradient pattern and a new dominance on what was formerly a posterior region. Even relatively advanced posterior zoöids have not completely lost their "posterior" characteristics. There is evidently an antagonism between the dominance of the fully developed or advanced head and the development of the new head and the new dominance in a new zoöid. The old nerve cords extend into and through the new zoöid, and the development of new cephalic ganglia and reorganization of the cords must attain at least a certain stage before the zoöid can become independent of the original dominance. Since the head region of the developing zoöid is attached to a more anterior region, it is incapable of independent motor activity. In the very early zoöid the new longitudinal gradient may not be distinguishable with certainty or may be visible only anteriorly, but there is undoubtedly activation internally in the region which becomes the head. The longitudinal gradient is, in general, less steep in the earlier zoöid than in the fully developed individual or the anterior zoöid of a chain, apparently because the developing head is more or less inhibited. Zoöids with advanced heads, separated experimentally, show rapid increase in motor activity and in rate of oxidation and reduction of indicators in the head and more anterior body-levels.

The gradient patterns of intracellular primary oxidation, reduction, and reoxidation, which have been regarded as representing differences in physiological condition in individuals and chains not injured by the experimental conditions, indicate quantitative graded differences in rate of metabolism involving respiratory factors. There seem to be good grounds for concluding that these gradients are correlated with differentials in rate of oxygen uptake and, consequently, with differentials in enzyme activity. The modifications of gradient pattern under particular experimental conditions, as described in preceding pages—e.g., with slow and slight oxygen increase after exposure to reduced dye, also primary oxidations with excess of hydrosulphite, and secondary or primary differential reductions in oxidized methylene blue and with the indophenol reaction—provide further evidence in support of this conclusion.

Two factors are apparently concerned in primary intracellular oxidation in dye solutions completely reduced by hydrosulphite. They are oxygen available in the body of the animal and oxidase activity. Assuming that these are the essential factors concerned, it appears that, if sufficient oxygen is available for intracellular oxidation of the indicator at all body-levels, differentials in oxidase activity determine the differentials in rate of oxidation and, consequently, the oxidation gradient. With excess of hydrosulphite and with slow and slight oxygen increase in the solution after a period in reduced dye, oxygen may diffuse out of the body so rapidly or enter so slowly that intracellular oxidation occurs more rapidly in regions of lower, than in those of higher, oxygen uptake. In these cases available oxygen rather than oxidase activity becomes the limiting factor in the rate of intracellular oxidation, and partial or complete reversal of the gradient pattern may result, or oxidation may occur only in those regions which oxidize less rapidly when more oxygen is available. Even in these cases the normal gradient pattern may appear with adequate aeration of the solution.

Secondary differential reduction of methylene blue following primary oxidation with the normal gradient pattern, observed frequently in methylene blue

reduced by hydrosulphite, progresses in the same direction as the normal reduction gradient and seems to indicate that, with continued exposure to dye solution reduced by hydrosulphite, available internal oxygen becomes insufficient to keep intracellular dye oxidized earlier in regions of higher, than in those of lower, oxygen uptake and oxidase activity. Reversal of the staining gradient in very low concentrations of oxidized methylene blue, either by intracellular reduction or, as certain experimental results requiring further confirmation suggest, irreversible differential decoloration of the dye in some other way, also suggest a quantitative gradient of physiological activity, decreasing in the same direction as the normal reduction gradient.

Whatever the conclusions concerning the relation of the gradient patterns to differentials in oxidase activity or in available oxygen, the indicator patterns are evidently associated with, and dependent on, quantitative physiological gradations of some sort. As regards their relations to fission, they appear to be essential factors in the origin and development of new zoöids. However, they give no evidence concerning the presence or absence or time of appearance of qualitative or specific features of pattern.

The strongly marked increase in rate of intracellular oxidation of indicators both anterior and posterior to a level of natural or experimental separation at a fission plane or elsewhere indicates the necessity for great care in determinations of oxygen uptake and carbon dioxide production on isolated pieces of organisms. The total effect of separation will be greater in short than in long pieces, since it extends for a certain distance from the level of separation and particularly since it occurs both anterior and posterior to the level of separation. If the wound closes rapidly, the effect of separation may soon decrease; but, if regeneration occurs, the new tissue oxidizes and reduces more rapidly than the old. In annelids and many other organisms, loss of blood or body fluids may alter physiological condition following separation of pieces. Even in the cleavage stages of the sea-urchin embryo, isolation of the basal (vegetal) blastomeres, e.g., of the 8-cell stage, is followed by more or less complete reconstitution of apical (animal) regions. In the light of the evidence on intracellular oxidation and reduction of indicators, not only in the forms considered in this paper but in many other organisms, the tacit assumption so often made that respiration remains unaltered in isolated pieces appears unjustified. Mention of one case concerned with an annelid appears in order here.

It was observed by Child and Rulon (1936) and noted in an earlier section of the present paper that in several species of earthworms two opposed gradients of Janus green reduction were present; but in one species, in which formation of new segments was apparently not continuing, only one gradient, decreasing posteropetally, was present. Somewhat later Maloeuf (1936) made determinations of oxygen uptake on very short pieces of an earthworm and maintained that no such pattern of oxygen uptake as the indicator reductions showed was present. Since no attempt was made in this case to compare these results with data from longer pieces and since loss of blood was virtually complete in the short pieces, it appears at least improbable that the differences in oxygen uptake in the pieces from different body-levels are the same as those in intact animals.

In this connection it is of interest that, by means of the nitroprusside reaction, Chu (1948) has found that in a microdrilous oligochete the -SH pattern shows relations to fission essentially similar in

general features to the normal oxidation-reduction indicator patterns.

It has been stated in earlier papers and is emphasized here that it is necessary to learn how to use the oxidation-reduction indicators with each organism serving as material. Observations on a few individuals with a few concentrations may lead to entirely incorrect conclusions. Differential toxic effects, with retardation or inhibition of reduction, have been regarded by some authors as the normal gradient patterns, though they are actually the reverse of the normal patterns. Attention was called to some of these in an earlier paper (Child, 1945*a*). With proper care in their use, the oxidation-reduction indicators unquestionably give evidence of the presence of quantitative physiological differentials in intact organisms. In the light of the evidence from many organisms, their correlation with quantitative differentials in metabolism or, speaking more specifically, with quantitative differentials in respiration appears beyond question. Moreover, these gradients are essential factors in development. The differential susceptibility of different gradient levels to depressing or inhibiting external agents makes it possible to decrease the gradient differentials experimentally, virtually to any degree, even to complete obliteration by means of single external agents. With such a decrease in early development, the course of development and the proportions of parts are altered in definite ways which are obviously correlated with gradient patterns, as has been repeatedly shown. With experimental obliteration of the gradients, all developmental evidence of polarity and symmetry is lacking, and axiate development does not occur, though the organism may live for days or even weeks. And, finally, new polarities and symmetries can be determined experimentally by quantitative external differentials and can be made visible by oxidation-reduction indicators before they become visible in morphogenesis.

SUMMARY

1. Rates of intracellular indophenol reaction, primary intracellular oxidation of dyes in reduced solutions, intracellular reduction of indophenol and oxidized dyes preceding appearance of toxic effects by the oxidized indicators, and reoxidation following reduction show essentially similar gradient patterns in their general relation to fission and development of zoöids in the flatworm, *Stenostomum*, and the oligochete, *Aeolosoma*, and the same pattern appears in primary oxidation in *Chaetogaster*.

2. In uninjured animals all the patterns, whether of oxidation or of reduction of indicators, show decrease in rate posteropetally from the head regions and often a slight increase in rate in posterior regions of individuals and zoöids. This posterior increase in rate is believed to be due in some cases to slight activation adjoining a fission plane, in one *Stenostomum* species probably to early stages of tail regeneration, and in *Aeolosoma* and *Chaetogaster* regeneration of segments probably plays a part.

3. In the earliest stages of developing zoöids the new longitudinal gradient pattern may not be distinguishable with certainty or may be evident only anteriorly. Each new zoöid originates from the posterior region of an individual or a preexisting zoöid. Its development involves the imposition of a new longitudinal gradient pattern on the region concerned, and this imposition occurs gradually with development of the new head region.

4. At all body-levels, rates of oxidation and reduction of the indicators decrease in the body-wall from ventral regions dorsally.

5. The fully developed head is more

susceptible to injury by intracellular oxidized indicators than other regions, with resulting retardation or complete inhibition of reduction and, with extreme injury, early disintegration.

6. Various modifications of gradient patterns occur under special experimental conditions, e.g., partial or complete reversal of the primary oxidation gradient in reduced dye solution with excess of sodium hydrosulphite; various degrees of reversal of the reduction gradient following differential injury decreasing posteropetally from the head; reversal of the reoxidation gradient with slight or slow oxygen increase following reduction.

7. In *N. paraguayensis*, which undergoes separation into varying numbers of pieces without morphological evidence of fission planes or new zoöids, the indicators give no evidence of predetermination of levels of separation.

8. In rapidly regenerating pieces of *N. paraguayensis* and in long individuals without recent regeneration, two opposed gradients of intracellular oxidation and reduction of indicators, varying in length, are present—one with decrease in rate posteropetally from the anterior end, the other with decrease anteropetally from the posterior end or from the region of segment-formation immediately anterior to the anal segment. In the long animals these gradients are usually separated by a middle region with little or no gradient. Rates of oxidation and of reduction also decrease from the ventral region dorsally.

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THE EFFECTS OF HOMOLOGOUS CARBAMATES ON THE RESPIRATION OF BLOCKED AND ACTIVE EMBRYOS*

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Among biological phenomena that have received considerable attention over a period of years, that of narcosis has held an extremely prominent position. Varied theories have been advanced for its explanation, and data upon which many of these theories have rested have come from results of experiments employing the homologous series of carbamates or urethanes. The effectiveness of these compounds as carcinogenic agents has also recently been emphasized, and rather marked differences in this activity as compared to their narcotic action have been pointed out (Larsen, 1947). The effects of carbamates on basic cellular activities, such as mitosis, have also shown them to be useful experimental tools in the analysis of many different cellular phenomena (Macht, 1948).

The present discussion has to do with results of extensive experiments on the effects of a homologous series of carbamates on the respiration of mitotically active and blocked embryonic cells of the grasshopper, *Melanoplus differentialis*.

MATERIALS AND METHODS

Embryos of known age and history were obtained from eggs of the grasshopper, *M. differentialis*, as previously pointed out (Bodine and Fitzgerald, 1948). Mitotically active as well as blocked (diapause) embryos were used, and all techniques employed were similar to those already described. The series of carbamates were chemically pure salts dissolved in Ringer's solution (pH 6.8) and added from the side arm of the respiration flasks. The carbamates were ethyl *n*-methyl, ethyl, *n*-propyl, *n*-butyl, *n*-amyl, and iso-amyl. Standard Warburg manometers with respiration flasks of 5-ml. capacity were used at 25° C. Large numbers of readings for each concentration of the different carbamates, using 100 embryos, were recorded and average curves drawn. Further details of techniques were similar to those recently reported (Bodine and Fitzgerald, 1948).

RESULTS

For all carbamates employed, no significant differences in response of mitotically active and blocked embryos were noted. However, the tendency for active cells to be more susceptible than blocked ones seemed evident. Therefore, data presented will apply equally to both cellular conditions.

Results of all experiments are graphically presented in Figures 1–6. An inspection of these figures shows that, in general, all carbamates in high concentrations produce a marked inhibition of the oxygen uptake of the embryos. Their relative effectiveness increases with each higher member of the homologous series.

The one and most outstanding exception in responses noted is the marked stimulation of oxygen uptake produced

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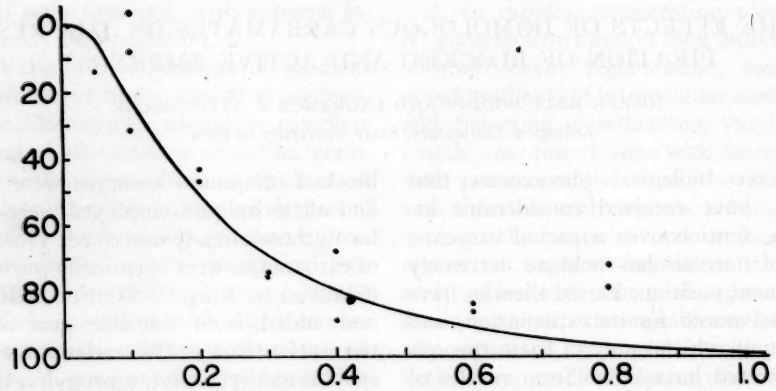

FIG. 1.—Shows the effect of ethyl *n*-methyl carbamate on the oxygen uptake of active and blocked embryos. Abscissa, concentration in mols per liter; ordinate, per cent inhibition. Curve represents average for all determinations; solid dots, average for minimum of 18 determinations in a single experiment.

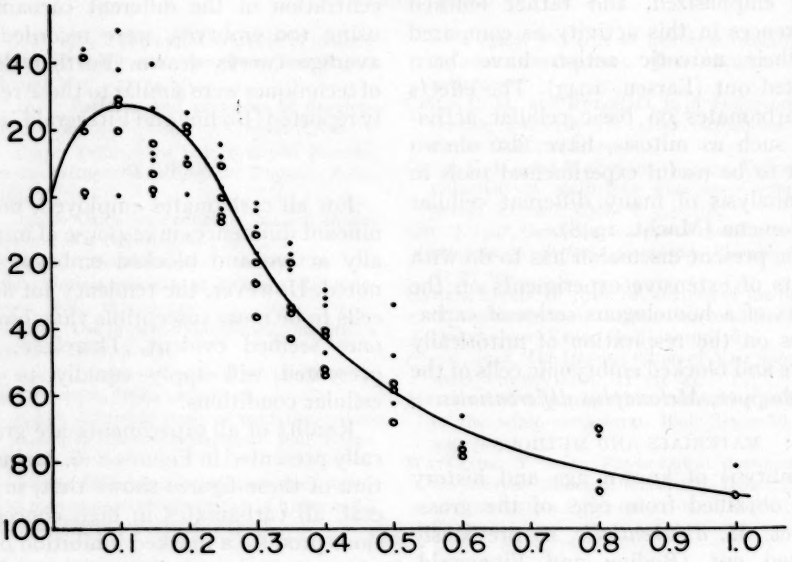

FIG. 2.—Same as Fig. 1, except for ethyl carbamate. Open circles, active embryos; solid circles, blocked embryos (from Bodine and Fitzgerald, 1948).

by low concentrations of ethyl carbamate. No other carbamate has been found consistently to give such a result with the methods employed, and, if such stimulation does occur for other members, it seems too transient to be detected by the present techniques.

The marked constancy of response during exposure to the reagents is shown in Figure 7 for ethyl *n*-methyl carbamate. Equilibrium in the degree of inhibition at specific concentrations seems quickly established and to continue over relatively long periods of exposure (Fig. 8).

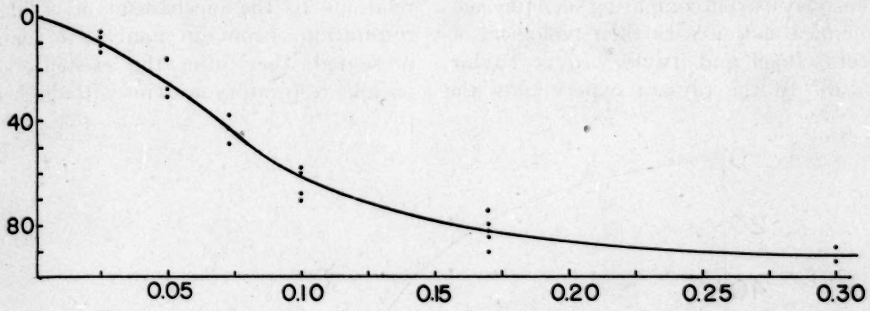

FIG. 3.—Same as Fig. 1, except for *n*-propyl carbamate

DISCUSSION OF RESULTS

As indicated above, many theories and ideas concerning the mode of action of narcotics, and especially carbamates, have been advanced. One objective of the present experiments was to obtain sufficient data of a quantitative nature on a relatively simple biological material in order to test the applicability of some of the existing theories of narcosis as applied to the reagents in question. That narcotics in high concentrations usually inhibit cellular phenomena seems well established, and most existing theories of narcosis have been developed around such a reaction. However, certain narcotics in low concentrations have been shown definitely to stimulate cellular reactions, and no one theory seems quite adequate as an explanation of such a result. Ethyl carbamate appears to be a reagent exhibiting this behavior to a marked degree (Taylor, 1936; Bodine and Fitzgerald, 1948), and, since no satisfactory explanation is at hand, the present discussion will be limited to the inhibitory phase of narcotic action.

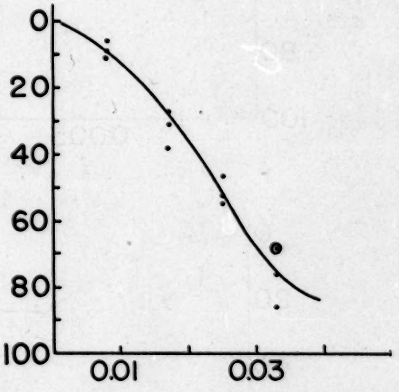

FIG. 4.—Same as Fig. 1, except for *n*-butyl carbamate.

The simple adsorption theory of Warburg (1921) perhaps has been most generally accepted from a physicochemical point of view, but its applications to cellular reactions have not always been too satisfactory. When data from the

present experiments are treated according to this theory by use of the Freundlich and Langmuir adsorption isotherms, no satisfactory confirmation is obtained. The present data, therefore, agree rather closely in this respect with those for many other biological materials similarly tested (Taylor, 1936).

The relative effectiveness of series of homologous carbamates in lowering the surface tension of water has also been extensively used in comparing such physicochemical action with their biological effects (Boell and Taylor, 1933; Taylor, 1936). In the present experiments the relative effectiveness of the carbamates used is, within limits, approximately two to three times for each additional CH_2 group. Similar results for other biological materials have been reported but no further explanations advanced (Taylor, 1934).

During the past few years Fisher *et al.* (1943) have re-emphasized the effects of narcotics on different cell processes and especially as regards their relations to the mechanism of cellular respiration. From an analysis of data presented, they infer the existence of parallel respiratory systems with marked

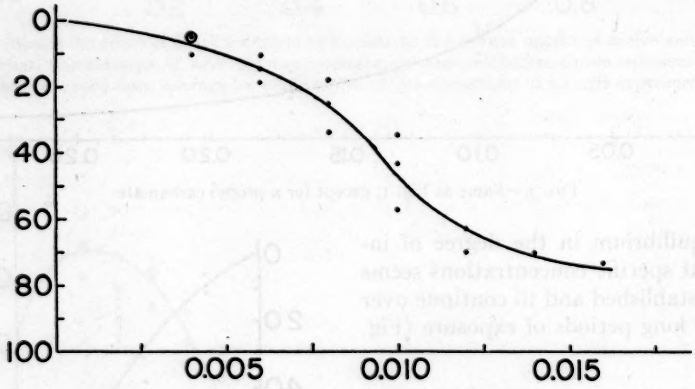

FIG. 5.—Same as Fig. 1, except for *n*-amyl carbamate

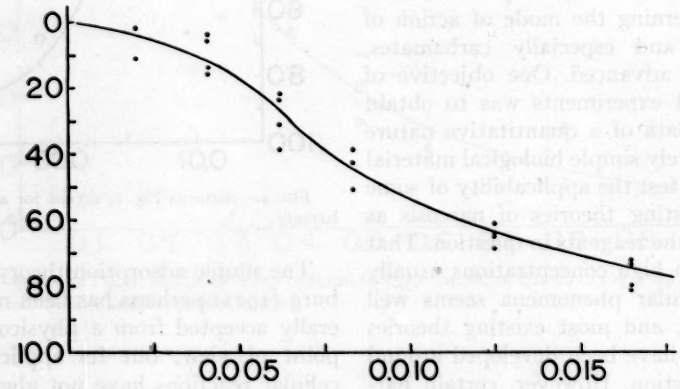

FIG. 6.—Same as Fig. 1, except for iso-amyl carbamate

differences in susceptibility to narcotics. For the grasshopper embryo it has been pointed out (Bodine and Fitzgerald, 1948) that, for ethyl carbamate in inhibitory doses, no such marked differences in response were apparent. For the other members of the homologous series of carbamates herein reported, a somewhat similar result is found.

In a recent paper by Brink and Posternak (1948) attention has been directed to a thermodynamic analysis of the relative effectiveness of certain narcotics, including the carbamates. These authors conclude that carbamates produce equal degrees of narcosis at the same value of thermodynamic activity. By such an analysis, "the principles of thermodynamics are used to supplement the limitation of experimental methods which do not measure the activity of narcotic at the cellular site of narcosis." By use of the formula, $A_{nar} = fN_{nar}$, where A_{nar} equals the thermodynamic activity of

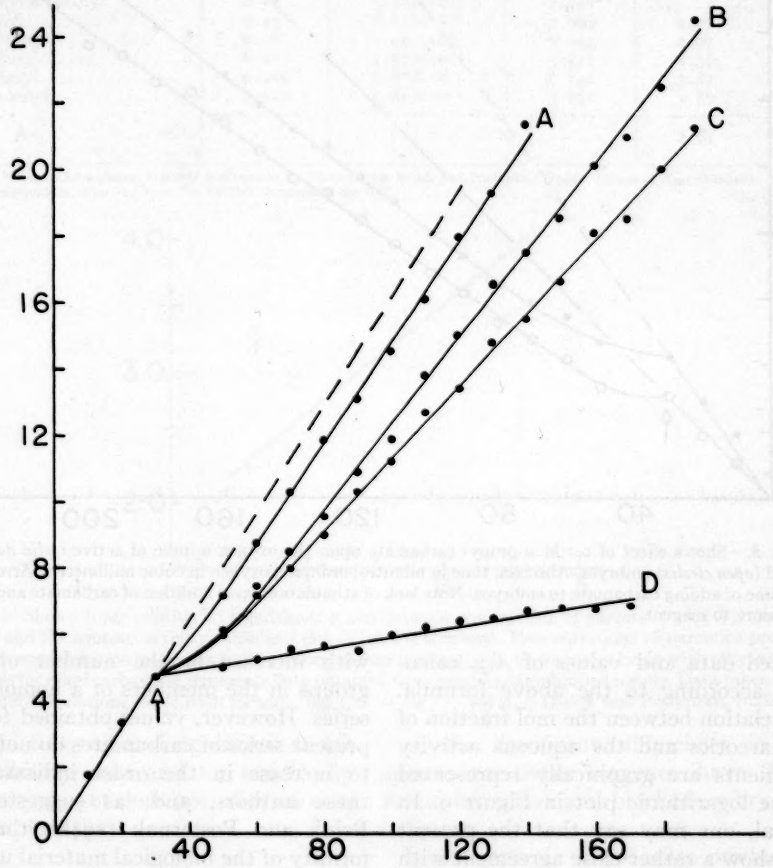

FIG. 7.—Shows effect of ethyl *n*-methyl carbamate on the oxygen uptake of active embryos. Abscissa, time in minutes; ordinate, oxygen in cubic millimeters. Arrow indicates time of adding carbamate to embryos. A = 0.01 M, B = 0.05 M, C = 0.10 M, D = 0.60 M. Note lack of stimulation upon addition of carbamate, also relative constancy of oxygen uptake during exposure to reagent.

the narcotic, f the activity coefficient of the narcotic in the aqueous phase, and N_{nar} equals the mol fraction of the narcotic in the solution bathing the narcotized cell, it is possible to calculate values from the present data and to test out such an idea. In Table 1 are summarized data and values of A_{nar} calculated according to the above formula. The relation between the mol fraction of the narcotics and the aqueous activity coefficients are graphically represented by the logarithmic plot in Figure 9. In general, one may say that the present data show a rather close agreement with the ideas as expressed by Brink and Posternak (1948) and that the thermodynamic activity appears to be a most satisfactory method for expressing narcotic efficiency.

Ferguson (1939) and Badger (1946), who also have worked along the lines of a thermodynamic analysis of narcosis, show that the thermodynamic activity for liminal narcosis increases regularly with increase in the number of CH_2 groups in the members of a homologous series. However, values obtained for the present series of carbamates do not seem to increase in the order indicated by these authors, and, as suggested by Brink and Posternak (1948), the uniformity of the biological material used in the present experiments may well account for the lack of such increases in values of the thermodynamic activity.

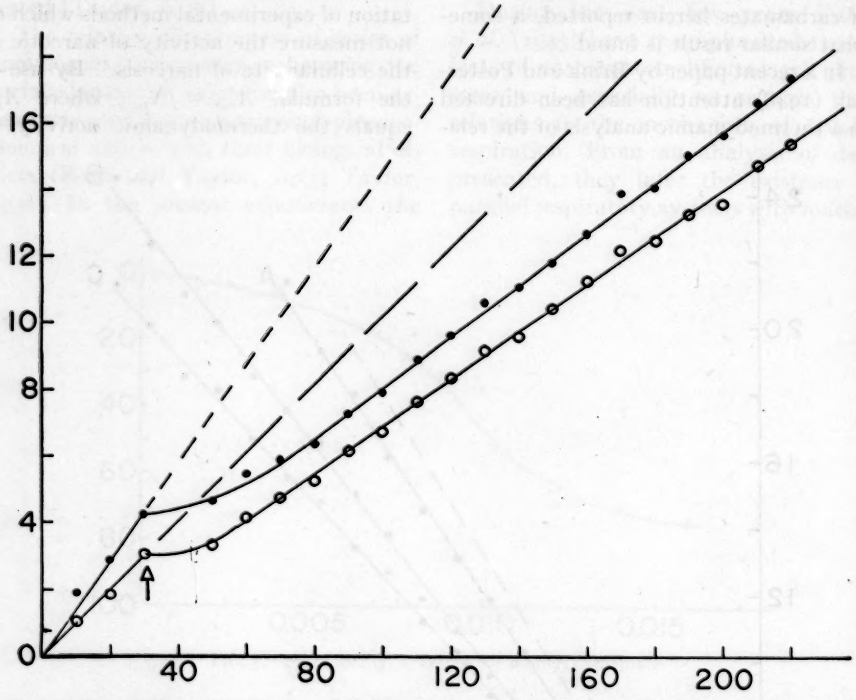

FIG. 8.—Shows effect of 0.1 M *n*-propyl carbamate upon the oxygen uptake of active (*solid dots*) and blocked (*open circles*) embryos. Abscissa, time in minutes; ordinate, oxygen in cubic millimeters. Arrow indicates time of adding carbamate to embryos. Note lack of stimulation upon addition of carbamate and length of exposure to reagent.

However good any theory is as an explanation for the inhibitory effects of narcotics in general, it is well to keep in mind the fact that some narcotics in low concentrations also produce stimulating effects just as real and significant as are the inhibitory ones and for which any one theory as yet does not offer an adequate explanation.

mates (ethyl *n*-methyl, ethyl, *n*-butyl, *n*-propyl, *n*-amyl, iso-amyl) on the oxygen uptake of mitotically active and blocked embryos of the grasshopper, *M. differentialis*.

TABLE 1*

CONCENTRATIONS OF CARBAMATES TO PRODUCE A 50 PER CENT INHIBITION OF OXYGEN UPTAKE BY ACTIVE AND BLOCKED EMBRYOS

Carbamate	Conc. (mol/liter) (50 Per Cent Inhibition)	Mol Fraction (Calc.) (N)	$-\log N_n$	$A_{nar} \times 10^2$
Ethyl-*n*-methyl	0.20	3.58×10^{-3}	2.446	.....
Ethyl	0.45	8.05×10^{-3}	2.094	2.97
n-Propyl	0.08	1.44×10^{-3}	2.842	2.07
n-Butyl	0.025	4.50×10^{-4}	3.347	2.38
n-Amyl	0.010	1.81×10^{-4}	3.742	3.87
Iso-amyl	0.010	1.81×10^{-4}	3.742	3.87
Av.	.....	.....	.....	2.84

* Values for aqueous activity coefficients, $f\infty$, taken from Brink and Posternak (1948). Values of A_{nar} obtained from equation, $A_{nar} = fN_{nar}$. For further description see text.

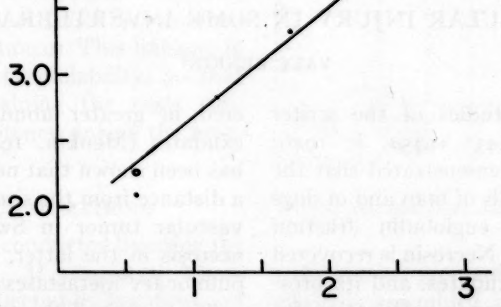

FIG. 9.—Shows linear relation on logarithmic scales between mol fraction of narcotic ($-\log N_n$) (on the ordinate) and the aqueous activity coefficient ($\log f$) (on the abscissa). Concentrations of narcotics producing 50 per cent inhibition in oxygen uptake taken as degree of narcosis. Carbamates begin with ethyl at left. Open circle for ethyl carbamate represents data obtained from previous unpublished results. Data taken from Table 1 and calculations made from formula, $\log 1/N = \log f - \log A_{nar}$ (Brink and Posternak, 1948).

SUMMARY AND CONCLUSION

1. A study has been made on the effects of a homologous series of carba-

2. No significant differences in response to these reagents have been found for active and blocked embryonic cells.

3. In general, all reagents produce a marked inhibition in the oxygen uptake. Ethyl carbamate in low concentration seems to produce a marked and con-

sistent stimulation, while all other compounds tested by the present methods did not produce such results.

4. A discussion of the present results, in view of the many existing theories of narcosis, is given.

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CELLULAR INJURY IN SOME INVERTEBRATES[1]

VALY MENKIN[2]

THE earlier studies of the writer (Menkin, 1943; 1945*a*, *b*; 1946; 1947) have demonstrated that the severely injured cells of man and of dogs liberate a toxic euglobulin fraction termed "necrosin." Necrosin is recovered in inflammatory exudates, and its presence offers a reasonable explanation for the pattern of injury in inflammation. The identification of necrosin has been confirmed by Smith and Smith (1945) and by Tanturi and his collaborators (1945). This substance is usually recovered in greater abundance from acid exudates (Menkin, 1945*b*). Recently it has been shown that necrosin injected at a distance from the site of a spontaneous vascular tumor in Swiss mice induces necrosis in the latter, as well as in the pulmonary metastases (Menkin, 1948*b*).

The object of the present report is to list evidence indicating that the severely injured tissues of invertebrates likewise liberate a toxic material, which, in turn, upon injection shortens the longevity of the soft-shelled sea clam, *Mya arenaria*. Necrosin is thermolabile (Menkin, 1943). This seems to be true, in large part, of the toxic material derived from the severely injured tissue of the invertebrate forms studied. Caspari (1926) has pointed out the possible presence of necrohormones associated with tumor tissue.

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Heilbrunn and Daugherty (1935) have shown that the cells of *Elodea* leaves exposed to ultraviolet rays produce substances which have an effect on other nonirradiated cells. Heilbrunn and Young (1935) also exposed sea-urchin eggs to Roentgen rays in the presence of ovarian tissue. They concluded from their observations that ovarian cells when exposed to Roentgen rays produce a substance or substances which act on the eggs, so that the effect is more pronounced than when the eggs alone are irradiated. Heilbrunn and his collaborators (1946) have recently studied, in particular, tissues subjected to heat. From such heated tissue they believe that there is liberated a dialyzable heat-stable toxic substance. The possibility that this substance is associated with potassium is pointed out, although it is also stressed that this is not the only factor involved. Furthermore, the toxic substance of heat-injured tissue may at times be combined with protein substances. This linkage, in turn, induces a thermolability, so that the extract containing the toxic substance loses its potency during the heating procedure.

MATERIAL AND METHODS

This study was conducted by using the soft-shelled clam, *M. arenaria*. These mollusks were collected at low tide and kept in the laboratory, usually in groups of 2 or 3 in finger bowls, each bowl being about 10 cm. in diameter. Rarely were these kept in groups of 4. Fresh sea water in the bowls was changed daily, but running sea water was not available. No other nutrient was provided. In this way the clams usually maintained a viable state from about 5 days to 1 week.

In a series of experiments collected in Table 1, all the organs of the clam were crushed in a mortar, the tissues being ground to a pulp. This was done under sterile conditions. When a viscous pulp was obtained, about 10 cc. of saline (0.6 per cent concentration)[3] were added. The syphonal muscles of several clams were then injected with 0.2 cc. each of this material. As controls, normal clams merely had their syphonal muscles pierced with a 24- or 25-gauge needle.

TABLE 1

EFFECT OF WHOLE EXTRACT OF MASHED TISSUE OF *M. arenaria* ON NORMAL *M. arenaria*

Experiment No.	Total No. Clams Receiving Whole Extracts of Organs of *M. arenaria* A	Total No. Untreated Clams, with Syphonal Muscle Pierced B	Survivors in Period Ranging from Several Hours to 2–3 Days Following Injection of Extracts or Merely Piercing Syphonal Muscle	
			A	B
A-1	3	2	0	2
B-1	3	2	1	1
C-1	3	2	0	1
D-1	2	2	0	2
E-1	3	3*	0	3
F-1	3	3*	0	3
Total	17	11	1	9
Percent alive			5.9	81.8

* The same clams were used as controls in Experiments E-1 and F-1.

In another group of clams, the data for which is assembled in Table 2, various protein fractions of the extract were employed. The original tissue extract was treated with $(NH_4)_2SO_4$ at one-third saturation. Centrifugalization for several minutes was then performed, and a fairly good separation was obtained. The supernatant phase presumably constituted the combined albumin and pseudo-globulin fractions of the extract, which were dialyzed in a Cellophane tube against

[3] This is a considerably hypotonic solution for clams; but this is perhaps of aid in extracting the toxic factor from these sea mollusks.

tap water. The precipitate was, in turn, taken to represent the euglobulin fraction of the extract, and accordingly it was dialyzed against tap water. The dialysis of both fractions lasted about 1 day when the SO_4= were found to be absent. About 0.2 cc. of these extracts were then injected into the syphonal muscle of clams. As controls, groups of clams again had their syphonal muscle merely pierced. While not in use, the extracts, which had been obtained under sterile conditions, were kept in a cold room.

The approximate intervals in hours of clams treated with the various protein fractions of the mashed extract of clam are listed in Table 3. In four experiments the crushed extract of the gills of *Limulus polyphemus* and the protein fractions were utilized and injected into the syphonal muscle of normal clams. The protein fractions of the blood of the horseshoe crab served as control for the gill foreign protein injected into the clams.

The effects of heating the extract or the protein fractions to a boil for 20 minutes to ½ hour are assembled in Table 4.

Finally, the extract of the clam tissue and some of its protein fractions were compared with the effect on clams of canine necrosin and canine leukocytosis-

TABLE 2

EFFECT OF PROTEIN FRACTIONS OF EXTRACTS OF TISSUES OF SOME INVERTEBRATES ON *M. arenaria*

Experiment No.	Total No. Clams Injected with Euglobulin Fraction of Tissue Extract A	Total No. Clams Injected with Combined Pseudo-globulin and Albumin Fractions of Tissue Extract B	Total No. Untreated Clams, with Only Syphonal Muscle Pierced C	Survivors in Period Ranging from Several Hours to 2–3 Days Following Injections of Protein Fractions, or Merely Piercing Syphonal Muscle		
				A	B	C
1	3*	3*	2	2	3	2
2	3*	3*	2	1	3	2
3	3*	2*	0	0	2	.....
4	3†	3*	2	1	2	2
5‡	3	3	2	1	3	2
6	3	3	2	1	3	2
7	3	3	2	0	1	1
8	3	3	2	1	2	2
9	3	3	2	3	2	2
10	3	3	.....	1	3	.....
11	3	3	?	0	2	.....
12	3	3	.....	0	0	.....
13	4	4	3	0	1	3
14	3	3	2	0	3	2
15	3	3	2	1	3	2
Total	46	45	23	12	34	22
Per cent alive	.....	.....	.....	26	80	95.7

* In these experiments the euglobulin fraction of the mashed gill tissue of *L. polyphemus* was employed on the clams. As a control for the foreign protein of *Limulus* in clams, the euglobulin of the blood of *Limulus* was employed. This is listed in col. B with an asterisk.

† This fraction was not found so effective. Contamination leading to putrefaction seemed to have reduced its potency, as indicated by the length of survival (3 days).

‡ The results are shown 1 day after administration of the extracts. Within 2 days both A and B had all succumbed.

TABLE 3

APPROXIMATE INTERVAL BETWEEN TIME OF INJECTION AND DEATH OF CLAM

Experiment No.	Survival Time of Clams Treated with Euglobulin Fraction of Mashed Tissue (Hours and Minutes) A	Survival Time of Clams Treated with Combined Pseudo-globulin and Albumin Fractions of Mashed Tissue (Hours and Minutes) B	Survival Time of Controls (with Only the Syphonal Muscle Pierced) C
1*	19:00 30:00 51:30	67:00 67:00 67:00	Over 67 hours Over 67 hours .....
2*	1:00 1:00 8:00	8:00 8:00 8:00	8:00 8:00 .....
3*	2:00	Over 16 hours Over 16 hours	....
4†	33:00 80:00 80:00	80:00 96:00	96:00 Over 96 hours .....
5	21:00 21:00 ±48:00	±48:00 ±48:00 ±48:00	69:00 69:00 .....
6	9:00 9:00 ±24:00	±24:00 ±24:00 ±24:00	±24:00 ±24:00 .....
7	2:00 23:00 23:00	23:00 23:00 Over 23 hours	? 23:00 .....
8†	19:00 49:30 Over 68 hours	42:00 68:00 Over 68 hours	Over 68 hours Over 68 hours .....
9†	Over 68 hours Over 68 hours Over 68 hours	50:00 68:00 68:00	Over 68 hours Over 68 hours .....
10 (faint odor of putrefaction)	20:00 20:00 ±48:00	27:30 ±48:00 ±48:00	20:00‡ 20:00 .....
11	3:30 3:30 7:00	3:30 ±24:00 ±24:00	Over 31 hours ....
12	3:00 9:00 9:00	9:00 9:00 9:00	Over 9 hours ....
13	10:30 28:00 28:00 34:00	10:30 22:30 27:00 46:30	46:30 55:00 55:00 .....
14§	28:00 28:00 28:00	42:00 66:30 71:00	66:30 90:00 .....
15§	28:00 42:00 50:30	47:00 50:30 50:30	66:00 114:00 .....

* Material injected obtained from crushed gills of *L. polyphemus;* in all other observations material used obtained from mashed organs of soft-shelled clam, *M. arenaria.*

† Extracts used had become contaminated and were putrid.

‡ Shell of clam partly broken.

§ Clams reinjected once with extracts.

TABLE 4

EFFECT OF HEATING THE TOXIC FACTOR ON *M. arenaria*

Experiment No.	Total No. Clams Injected with Unheated Clam Extract or Its Protein Fractions	Total No. Clams Injected with Heated Clam Extract or Its Heated Protein Fractions	Controls (Untreated Clams, with Syphonal Muscle Pierced)	No. Clams Surviving from Several Hours to 2 Days Following Administration of Unheated Material	No. Clams Surviving from Several Hours to 2 Days Following Administration of Heated Material	No. Clams Surviving from Several Hours to 2 Days Following Piercing of Syphonal Muscle
A*	2	2	2	1	0	2
B*	2	2	2	0	1	1
C†	3	3	3	0	3	3
D†	2	2	2	0	1	2
E	3	3	3	0	3	2
F	2	2	2	0	2	2
G	2	2	2	0	1	2
Total	16	16	16	1	11	14
Per cent alive	.....	.....	.....	6.2	68.8	87.5

* Total globulins of clam used; in some of the other observations either the whole extract of the clam or the euglobulin fraction was utilized.

† Whole extract of clam used; in experiments E, F, and G the euglobulin fraction of clam extract was used.

promoting factor. Whereas necrosin is, or at least is associated with, the euglobulin fraction of inflammatory exudates (Menkin, 1943, 1945*a*), the leukocytosis-promoting factor of canine exudates is recovered in the α_1 and α_2 globulin fractions of exudates (Menkin, 1947). The data on this set of experiments appear in Table 5.

out of 17 experimental clams survived, or 5.9 per cent (cf. col. A). On the other hand, 9 out of 11 controls survived within the same interval, which represents 81.8 per cent. It would therefore appear as if there is some toxic factor liberated in the extract of the mashed tissue of *M. arenaria*.

TABLE 5

SURVIVAL TIME OF CLAMS IN VARIOUS TYPE EXPERIMENTS

Experiment No.	Total No. Clams Studied	Type of Material Injected	Longevity (Days)	Experiment No.	Total No. Clams Studied	Type of Material Injected	Longevity (Days)
D	2	Whole clam extract	2	N-3	2	Canine necrosin of exudates	4
D_1	2	Heated whole clam extract	9	LPF-3	2	Canine leukocytosis-promoting factor of exudates	8
D_2	2	Controls	13				
G	2	"Euglobulin" of clam tissue extract	1	C-3	2	Controls	11
G_1	2	Heated euglobulin of clam tissue extract	2	N-5	2	Canine necrosin of exudates	2
G_2	2	Controls	4	LPF-5	2	Canine leukocytosis-promoting factor of exudates	3
F	2	Euglobulin of clam tissue extract	1	C-5	2	Controls	5
F_1	2	Heated euglobulin of clam tissue extract	8	N-6	2	Canine necrosin of exudates	2
F_2	2	Controls	7	LPF-6	2	Leukocytosis-promoting factor of exudates	3
E	3	Euglobulin of clam tissue extract	2	C-6	2	Controls	6
E_1	3	Heated euglobulin of clam tissue extract	6	N-4	2	Canine necrosin of exudates	5
E_2	3	Controls	5	LPF-4	2	Canine leukocytosis-promoting factor of exudates	3
C	3	Whole clam tissue extract	2	C-4	2	Controls	7
C_1	3	Heated whole clam tissue extract	5				
C_2	3	Controls	7				

RESULTS

When the syphonal muscles of *M. arenaria* were injected with the whole extract of the mashed tissue obtained from another normal clam, the mollusks survived for a definitely briefer period than did the control animals. The data on a series of such experiments appear in Table 1. It is seen that in a period ranging from several hours to 2 or 3 days, 1

This toxic factor seems to be located primarily in the fraction of the extract obtained by treating the latter with saturated $(NH_4)_2SO_4$ at one-third saturation. This, as a rule, yields what is termed the "euglobulin protein fraction" (after dialysis of the sulphate ions). The residual protein fraction represents the combined albumin and pseudo-globulin fractions of the mashed tissue extract. A

glance at Table 2 indicates that, whereas 95.7 per cent of controls survived from several hours to 2–3 days (col. C), only 26 per cent survived in the group of clams treated with the euglobulin fraction (col. A) and 80 per cent in the group treated with the combined albumin and pseudo-globulin fractions of the extract (col. B).

A more detailed analysis of the survival time in such treated clams is shown in Table 3, where the approximate interval in hours is tabulated. The euglobulin fraction induced a survival time ranging between 1 and 51½ hours. If, however, the protein fraction became putrid, as it did in several experiments, its toxicity was considerably reduced. Clams treated with such decayed euglobulin fraction survived between 19 and 80 hours. The combined pseudo-globulin and albumin fractions used in this study never became putrid. The span of survival in clams treated with this fraction ranged between 3½ and 96 hours. This is roughly about twice as long as the nonputrid euglobulin fraction. The control clams survived for even longer intervals, ranging between 8 and 114 hours. Essentially the same effect was obtained when, instead of clam extract, different fractions of mashed gill extracts of *L. polyphemus* were administered to *M. arenaria* (Table 3).

An attempt was made to determine some of the properties of the extract of tissues of *M. arenaria*. The pH of the extract was found to be about 6.0. The euglobulin fraction when obtained had a pH of about 6.5. The biuret reaction on the fractions used was positive. Over two hundred clams were studied in the course of this study. An endeavor was made to determine the relative thermolability of the fractions. It was found that boiling the euglobulin fraction or the total globulin fractions or the whole extract definitely decreased the toxicity of the factor. The data are assembled in Table 4. It is seen that in the series studied, whereas, in the time studied, only 6.2 per cent survived the administration of the unheated material, heating the whole extract or any of the globulin fractions decreased the toxicity. The result is a greater number of survivors, so that as much as 68.8 per cent of clams survived administration of the heated material from several hours to 2 days. Since 87.5 per cent of the controls survived, as compared to 68.8 per cent in the heated group, this would possibly indicate that, in addition to the thermolabile toxic factor, there is also in the extract a smaller amount of a thermostable toxic substance.

Finally, a comparative study was made of the effect of a vertebrate toxic material recovered from inflammatory exudates of dogs and termed "necrosin" (Menkin, 1943). Necrosin, as mentioned above, is found in the euglobulin fraction of particularly acid exudates. It is seen in Table 5 that canine necrosin, just like the whole extract of the crushed tissues of a clam or the derived euglobulin fraction, shortens the life-span of *M. arenaria* when compared to the length of survival of control untreated clams. The writer has also shown that in the pseudo-globulin fraction of canine exudates there is a polypeptide capable of increasing the number of circulating white cells in a dog (Menkin, 1948*a*). This material (abbreviated as the "LPF") is apparently, in contrast to necrosin, not toxic to the tissues of the dog. When tried in clams, the LPF, or leukocytosis-promoting factor of canine exudates, is found to be, as a rule, definitely less toxic than necrosin (Table 5).

DISCUSSION

The foregoing study indicates that the severely injured tissues of some invertebrates, notably *M. arenaria* and *L. polyphemus*, contain a toxic factor. This toxic factor seems to be located primarily in the protein fraction obtained by treating the extract of the injured tissues with $(NH_4)_2SO_4$ at one-third saturation. In vertebrate material this constitutes the euglobulin fraction. Earlier studies by the writer indicate that the products of cell injury in the dog, as exemplified in inflammatory exudates, contain a similar toxic material, termed "necrosin" (Menkin, 1943; 1945*a*, *b;* and 1946). Necrosin is found to be thermolabile. This is precisely what has been found to be the case in the extract of the crushed tissue of *M. arenaria* (Table 4). It therefore would seem reasonable to the writer to suppose that a toxic euglobulin can be recovered from severely injured cells, in general, whether one is dealing with vertebrate or invertebrate material.

The fact that boiled material yields a somewhat more toxic effect than is found in untreated control clams (Tables 4 and 5) possibly indicates that, in addition to the toxic thermolabile factor of the severely injured tissue of *M. arenaria*, there is also present a thermostable factor. This possibility is being investigated further.

CONCLUSIONS

The severely injured cells of vertebrate material seem to release in inflammatory exudates a toxic material located or associated with the euglobulin fraction of exudates. This toxic substance, which, per se, offers a reasonable explanation for the basic pattern of injury in inflammation, has been termed "necrosin."

What appears to resemble this state of affairs is likewise encountered in severely damaged tissues of some invertebrates. Crushed tissues of either *M. arenaria* or the gills of *L. polyphemus* yield an extract which is toxic to otherwise normal *M. arenaria*. The toxicity seems to reside primarily in the protein fraction obtained by treating the extract with $(NH_4)_2SO_4$ at one-third saturation. This chemical procedure in vertebrate material yields, after dialysis of the sulphate ions, what is termed the "euglobulin fraction." It is in association with the euglobulin fraction of inflammatory exudates that necrosin is recovered.

The toxic euglobulin factor in the injured tissue of the invertebrates studied is thermolabile. Boiling tends to inactivate it in large part. This is a similar property possessed by necrosin.

The studies indicate and suggest that the toxic factor in the severely injured tissues of the invertebrate forms studied is similar to, if not identical with, necrosin recovered from the products of cellular injury in vertebrates.

There may be an additional thermostable toxic factor in the injured tissues of *M. arenaria*. This possibility, however, requires further study.

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REGENERATION OF THE OPTIC NERVE AFTER SECTION WITH RETURN OF VISION IN THE CHARACIN ASTYANAX MEXICANUS

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STONE and his collaborators (1934, 1937) performed a series of interesting experiments involving the reimplantation and transplantation of eyes in amphibians. Return of vision in these animals was obtained by regeneration of the lens, retina, and optic nerve. In 1940, Stone and Sapir performed lens-extirpation experiments on several species of amphibians and one species of fish, *Fundulus heteroclitus*. There was no regeneration of the lens and consequently no return of vision in the teleost. No information is given by these authors about the fate of the optic nerve. Earlier experiments on reimplantation of eyes and on regeneration of the lens were performed chiefly on amphibians. Koppanyi did successful reimplantation of eyes in *Carassius vulgaris* Nilss [=*Carassius carassius* (Linnaeus)]. He described a return of light sensitivity after his operations. There has been some dispute over the question of lens regeneration in teleosts, but the absence of such a phenomenon has been confirmed in *F. heteroclitus* by Morrill (1906) and by Stone and Sapir (1940). Sperry (1948) obtained return of vision in several species of marine teleosts after crushing the optic nerve. In this case the nerve fibers were reported as entirely separated, but the sheath of the nerve remained intact, leaving a pathway for the regenerating fibers.

Since return of vision was noted in two *Astyanax mexicanus* (Filippi) after complete section of the optic nerve incident to another experiment, further work was undertaken. More operations of a similar character were performed, together with reimplantations and implantations of eyes from the eyed *Astyanax* into the eye sockets of its blind cave derivatives, *Anoptichthys jordani* Hubbs and Innes and *An. hubbsi* Alvarez. Some individuals of the blind species have a remnant optic nerve (Breder and Rasquin, 1947; Gresser and Breder, 1940; Breder and Gresser, 1941; Breder, 1944).

A solution of 1 per cent urethane was used for anesthesia. In the operations in which the eyes were reimplanted, the entire eye was removed form each side in turn and then replaced in the same socket from which it had come. In the case of transplantations, two fish were operated on at once, with the eyes from the same sides exchanged. Care was taken to ori-

ent the eyes normally. Where the optic nerves were merely severed, an incision was made in the dorsal part of the soft tissue surrounding the eye, the eye was moved outward laterally, and the optic nerve was completely cut with iridectomy scissors. In many cases the blood vessel supplying the eye was inadvertently also cut. This last method left the more ventral eye muscles intact and held the eye in place, so that the cut ends of the nerve approximated one another. In reimplanting and transplanting, it was difficult not only to place the eye properly in the socket but to keep it in place thereafter, as the fish had to be returned to water immediately, leaving no chance for healing in place to occur before the fish was actively swimming again. This difficulty seemed to be one reason for nonreturn of vision in most animals with reimplanted eyes.

In most cases small eyes from young fish were used for implanting in the blind forms, for the eye socket in the blind fish is considerably smaller than that of the eyed form.

One *Astyanax* which had had both lenses extirpated 2 years previously is also included in this report.

Out of 42 fishes, 2 died as a result of the operations, 1 with severed optic nerves and 1 with reimplanted eyes. Otherwise, the fish remained in good health, eating regularly and showing pugnacity when other fish passed too closely; that is, they moved sharply toward the passing fish as if to attack.

For histological examination, the heads of the fish were partly dissected until the optic nerve was exposed. They were fixed in 10 per cent formalin, imbedded in paraffin, sectioned at 15 μ, and stained, using Weigert's method for medullated fibers.

Table 1 gives the numbers and kinds of fish used, the operations, and the results.

Vision returned to the animals with severed optic nerves in about 4 weeks. A few days prior to this time, the dark pigmentary phase common to blinded fishes was lost, and the normal lighter phase became apparent. Next, the fish reacted to the shadow of a hand passed across the front of the tank, and, finally, the fish were observed to strike accurately at falling particles of food. Figures A and B in Plate I show sections from the eyes of the same fish, both optic nerves of which had been severed. One eye, represented by Figure A, did not regain vision, and the degenerated condition of the fibers can be seen in the photograph. Vision was regained in the other eye, and Figure B shows the heavily myelinated fibers characteristic of the functional nerve.

The one fish with reimplanted eyes that regained vision, however, never lost its dark pigmentary phase. The reason for this anomalous behavior of the pigmentary system is unknown. Such behavior is unique in the experience of the author and has apparently not been reported in the literature. The fish was certainly able to see, for accurate striking at falling food particles was observed 5 weeks after operation. Unilateral tests made by passing a hand before the glass side of the tank showed that the fish reacted sharply, no matter which eye was exposed to the stimulus. The histological sections proved that both eyes of this fish had myelinated optic nerves, so that vision was regained by both eyes. Figure C shows the optic nerve of one reimplanted eye from this fish.

It was found impossible to test the normal unoperated *Astyanax* in a nystagmus apparatus, for their only reaction to the experimental procedure was that of panic. However, it was possible to test

the operated fish whose vision had returned. After they had been left in the apparatus for 24 hours, they would remain sufficiently quiet for the eye movements to be observed. The eye that was functioning could be seen to follow the revolving stripes. The one fish that regained vision after reimplantation of the eyes was unable to move the eyes because the function of the oculomotor musculature had been disrupted. Consequently, when reacting to stimuli, this fish moved the entire body. Feeding reactions were found to be the best criterion for testing the return of vision in all the operated fishes. When blind, these fish feed from the bottom, finding food by the sense of smell and taste. With vision the feeding habits are entirely different; the fish strike accurately at particles floating on the surface or falling through the water. The only reason which can be advanced to account for the fact that these operated fish were more amenable to experimental procedures is that they were probably receiving a lesser number of retinal stimuli.

In some of the animals that did not regain vision the lens never became opaque, and the eyes remained in apparently perfect condition. In others the lens became opaque, while the eye was otherwise seemingly healthy; and in others the eye was completely cast off. In the operates with transplanted eyes all the eyes transplanted in *Astyanax* and the blind *An. jordani* were cast off, none became vascularized. The eyes transplanted into the blind form, *An. hubbsi*, were either cast off or absorbed. In the latter case the transplants were covered by regenerated dermal and epidermal tissues.

In one blind specimen into which a rather large eye had been implanted, the eye remained visible for several weeks, and some of the pigment migrated dorsally and ventrally out from the transplant into two conspicuous spots, which gradually disappeared. Billingham and Medawar (1948) published an extensive report on pigment migration in guinea-pig skin. In this case the pigment migrates from a black graft in the spotted guinea pig. They conclude that this process is carried out by an infective transformation of nonpigmented dendritic cells by pigmented ones. Moreover, the cells which have become pigmented remain so permanently. The blind fish are known to have dendritic melanophores in the skin with little or no pigment granules (Rasquin, 1947), which conceivably could pick up melanin granules from adjacent

TABLE 1

RESULTS OF EXPERIMENTS ON OPTIC NERVE SECTIONS

Fish	Age When Operated (In Months)	No. Operated	Type of Operation	Mortality	Regained Vision
As. mexicanus	12	1	Lens extirpated	0	0
As. mexicanus	18	12	Optic nerve severed	1	5
As. mexicanus	18 3	8 2	Eyes reimplanted	0 1	0 1
As. mexicanus	18 3	6 4	Eyes transplanted	0 0	0 0
An. jordani	12	3	Eyes transplanted	0	0
An. hubbsi	12	6	Eyes transplanted	0	0

pigment cells in a pigmented graft. However, the graft in this case was represented by an eye, and it appeared that the only chromatophores present must be confined to those scattered in the iris (Walls, 1942). There were scarcely enough melanophores in the transplant to produce the intense black spots in the host. Moreover, this condition was not a permanent one in the fish. It would seem that the choroid coat of the grafted eye rather than migrating melanophores provided the pigment which was subsequently phagocytized or inactivated by the host.

Melanophores have made their appearance in yellow goldfish as a result of experimental wounds and X-ray treatment (Smith, 1931, 1932; Ellinger, 1939, 1940), and these pigment cells disappear after a time. Trauma induced by the transplantation of the eye into the blind fish might cause such a melanophore reaction, but it is noteworthy that only one of the blind fish hosts exhibited this reaction. What melanin granules are present in the melanophores of the blind fish are not sufficiently numerous to give even a slight gray coloration to any other parts of the body.

The eyes of the one fish whose lenses had been extirpated became atrophied. Figure D shows the optic nerve to be degenerated in this case also. This fish had been living without lenses for over 2 years. Soon after the operation had been performed, this particular fish was known to be light-sensitive (Breder and Rasquin, 1947). In the ensuing time the eyes became atrophied, with complete degeneration of the retinal elements, which would account for the subsequent degeneration of the optic nerve.

Breder (1943) and Schlagel and Breder (1947) have noted a peculiar item of behavior in the *Astyanax*. An unknown stimulus will change the normally quiescent behavior of several fishes in a tank to one of active fighting. They will then chase one another and bite off pieces of fins. Some or all of the fish will die as a result of this behavior. It had been thought that overcrowded conditions might be the cause, but the phenomenon was observed in two fishes in which vision had returned. These two were isolated in a 15-gallon tank, and one was so badly beaten that it had to be sacrificed. The entire caudal fin was gone, the other fins were badly frayed, and lesions were noted on the body where scales were missing.

In agreement with Stone and Sapir's work (1940) and with that of earlier authors, no evidence of regeneration of the lens was shown by the fish used in the present study. No regenerated lens appeared after extirpation of the lenses, and, in fact, the optic nerves ultimately degenerated after this operation. In fishes operated on in such a way that the lenses remained in position, the lens continued to degenerate, once it had become opaque, and there was never any return of vision. Among the animals whose lenses remained clear after the operations, some regained vision and some did not. It appears that, in order to permit a return of vision after section of the optic nerve in these fishes, the cut ends of the nerve must approximate one another, so that the growth of interfering connective tissue cannot block the pathway for the regenerating nerve fibers, and that vascularization must take place before the lens and retinal elements degenerate. Failure to regain vision or light sensitivity in these characins is evidently caused by more than one factor, namely, the degeneration of the lens and retinal elements and the failure of regenerating fibers of the optic nerve to reach the brain.

PLATE I

SUMMARY

1. Return of vision was obtained in 5 of 12 *As. mexicanus* after complete section of the optic nerves.

2. Return of vision was obtained in 1 of 10 *As. mexicanus* after reimplantation of the eyes.

3. No return of vision was obtained in *As. mexicanus* after transplantation of the eyes or extirpation of the lenses, nor was any vision obtained in the blind cave derivatives of this species after implantation of the eyes.

4. Fishes in which the lenses degenerated showed no regeneration of the lens and no recovery of vision.

5. Fighting behavior was noted between two of the fish which showed a return of vision.

6. In order to have a return of vision in *As. mexicanus*, after section of the optic nerves or reimplantation of the eyes, the cut ends of the nerve must approximate one another in the wound, and vascularization must be established before the lens and retinal elements degenerate.

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PLATE I

FIG. A.—Site of the exit of the optic nerve from the eye of a fish in which the optic nerve had been severed, without subsequent return of vision.

FIG. B.—Site of the exit of the nerve from the eye of a fish in which the optic nerve had been severed, with subsequent return of vision.

FIG. C.—Site of the exit of the optic nerve from the eye of a fish in which both eyes had been reimplanted, with subsequent return of vision.

FIG. D.—Site of the exit of the optic nerve from the eye of a fish in which the lenses had been extirpated, without subsequent return of vision. Magnification × 100.

STUDIES OF THE DAILY RHYTHMICITY OF THE FIDDLER CRAB, UCA. MODIFICATIONS BY LIGHT[1]

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A PERSISTENT diurnal rhythm of color changes in the crab, *Uca*, was first reported by Abramowitz (1937), the animals darkening by day and lightening by night even when maintained under constant conditions of illumination. This rhythmical response was found by Brown and Webb (1947, 1948*a*, *b*) to involve activity of both the white and the black chromatophores and to continue in constant darkness for at least several weeks completely in phase with the solar day-night cycle. Furthermore, the frequency of the rhythm was found to be uninfluenced by temperature within the range of 6°–26° C. In the temperature range of 0°–3° C., however, the physiological processes underlying the rhythm were greatly retarded. *Uca* kept in constant darkness and exhibiting a normal rhythm had this rhythm thrown about 6 hours out of phase with the solar day-night cycle as a result of being kept at a temperature of 0°–3° C. for 6 hours; the rhythm then persisted in this new condition as long as the animals remained in darkness. In view of the persistence of the rhythm both in and out of phase with the solar cycle under conditions in which all diurnally varying factors known to influence the organism (temperature, light, and humidity) were held constant, as well as the retardation of the rhythm by low temperature, Brown and Webb assumed that the rhythm must be dependent in significant measure upon a series of metabolic events. This conclusion seemed difficult to reconcile with the lack of an observable influence of temperature, through a wide range, on the frequency of the rhythm in this poikilothermic organism. The only explanation appears to be that the mechanism for the daily rhythmicity in *Uca* possesses a remarkable capacity for accurate temperature compensation.

The chromatophore system of *Uca*, though governed primarily by that center within the organism responsible for the daily rhythmicity, is not influenced exclusively by it. It was clearly demonstrated by Brown and Sandeen (1948) that the immediate conditions of illumination and of temperature also contribute in determining the degree of dispersion of the pigment within the chromatophores. There has as yet been no elucidation of the route or routes through which light and temperature influence the coloration, whether (*a*) by direct action upon the chromatophores; (*b*) by reflex action upon the chromatophore system, bypassing the center of control of the daily rhythmicity; (*c*) by modifying the ability of the center of rhythmicity to influence the pigmentary system, through (*d*) modifications in the activity of the rhythmical center itself, or, finally, (*e*) by some combination of these routes.

Furthermore, there have been no reports as to whether the normal endoge-

[1] This investigation was supported by a research grant from the graduate school of Northwestern University.

nous 24-hour rhythm of *Uca* can be modified by alteration of the conditions of illumination of the animals and, if so, under what conditions this is possible and through what channels the external conditions of illumination are able to influence the center. It was believed that a knowledge of these characteristics of the daily rhythmicity of *Uca* could contribute much to our knowledge of the basic mechanism involved.

The experiments to be reported here are the results of attempts to modify the daily rhythmicity of color change in *Uca* by controlling the times, durations, and frequencies of periods of illumination. The data obtained are studied with the aim of determining the means by which synchronization of the phases of the endogenous rhythmicity with the solar cycle of day and night is normally effected and maintained.

EXPERIMENTS

The animals in all the following experiments were specimens of *Uca pugnax*, collected near Woods Hole, Massachusetts. They were usually kept in the laboratory in tanks supplied with shallow, running sea water. For each single experiment a group of ten animals was placed in an aluminum container which had been painted black on the inside and which contained sufficient water to cover the animals. For each group of experiments a control group of ten animals was removed from the laboratory tank at the same time as the experimental animals and maintained in similar containers in constant darkness for the duration of the experimental period. The chromatophores of all animals, control and experimental, were examined at 1:00 A.M., 7:00 A.M., 1:00 P.M., and 7:00 P.M. daily, and the state of the chromatophores was recorded according to the method of Hogben and Slome (1931). The average condition for ten animals was then determined. Therefore, each point on the graphs in this paper represents an average of ten animals.

Figure 1 shows the typical diurnal rhythm exhibited under conditions of constant darkness. The ordinate gives the degree of dispersion of pigment on the Hogben and Slome scale (5, complete dispersal; 1, complete concentration). The light and dark segments at the top of the graph and corresponding ones below represent time in days, the blackened segments indicating the interval 6:00 P.M. to 6:00 A.M., and the light segments

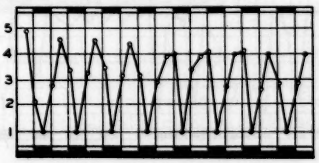

FIG. 1.—The normal diurnal rhythm of *Uca* kept in constant darkness. (See text for the interpretation of the figures.)

6:00 A.M. to 6:00 P.M. The lowermost band represents the experimental treatment; the light areas are periods of illumination and the dark ones periods of darkness.

Figure 2 shows the behavior of the chromatophores under conditions of reversed illumination, followed by constant darkness and, finally, by normal day-night illumination. Beginning at *A*, the animals were illuminated from 9:00 P.M. to 9:00 A.M. daily and kept in the dark from 9:00 A.M. to 9:00 P.M. The intensity of illumination was about 150 foot-candles. At *B* they were placed in continuous darkness. At point *C* they were placed in the laboratory, where they were in light during the daytime and in the dark at night. The maximum daytime illumination in the laboratory was probably not greater than 40 foot-candles.

Although the chromatophores show a marked degree of dispersion for the first 24 hours of the reversed illumination, it is obvious that such rhythm as is observed is correlated with the periods of light and darkness rather than with solar day and night. By the second day of the experiment a rhythm of normal amplitude is established, with the phases the reverse of those observed under normal conditions. During the period of constant darkness, begun at *B*, the reversed rhythm was maintained, although the amplitude was small for the first day.

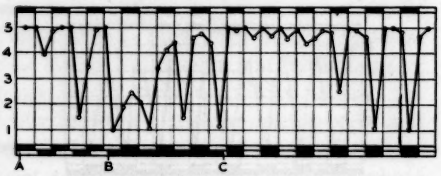

FIG. 2.—Beginning at *A* (9:00 P.M.), *Uca* were illuminated for 12-hour periods (9:00 P.M. to 9:00 A.M.) and kept in the dark for a similar period (9:00 A.M. to 9:00 P.M.). Beginning at *B*, they were kept in constant darkness. At *C* (3:00 P.M.) they were restored to the natural day-night environment.

The return of the animals to normal day-night light changes is followed immediately by an inhibition of the rhythm, with almost complete dispersal of the pigment, which persists for 3 days. At the end of this time a rhythm in phase with solar day and night appears. The difference in time required to establish the reversed rhythm as compared with that necessary to re-establish the normal is probably to be explained on the basis of the difference in intensity of illumination.

Figure 3 shows the condition of the chromatophores of a group of ten animals under the following conditions. A preliminary period of 30 hours in darkness, during which the state of the chromatophores was determined at the usual times. At the end of this period (at *A*) the animals were exposed to illumination of 80 foot-candle intensity from 7:00 P.M. to 7:00 A.M. daily and to darkness from 7:00 A.M. to 7:00 P.M. At point *B*, a reversed chromatophore rhythm having been established, the animals were placed in constant darkness. After 4 days (*C*), a series of 6-hour periods of alternating light and darkness was initiated. The animals were illuminated from 7:00 A.M. to 1:00 P.M. and from 7:00 P.M. to 1:00 A.M., and this treatment was continued to point *D*, when they were returned to constant darkness.

It is seen that under these conditions the time required for reversal of the rhythm is 3 days. The reversal is preceded by a 2-day period during which the chromatophores show a marked degree of dispersal at all times. During the period of constant darkness (between *B* and *C* on the graph) an increasing amplitude of the rhythm is developed, so that by the last day in darkness maximal degrees of concentration and dispersion are seen. The initial 6-hour period of illumination (beginning at *C*) occurs when the animals are entering the "night" phase of the rhythm, and the effect is immediately obvious. By the second day of this treatment the most concentrated condition of the chromatophores is recorded at 7:00 A.M.—a shift of 6 hours from that found in the previously established rhythm. Observations on these animals were made for only 1 day following their return to constant darkness (*D*). It is obvious, however, that they did not exhibit an immediate return to the rhythm previously established but still showed the most concentrated condition of the pigment to be at 7:00 P.M. The possible significance of this last fact will be discussed later.

A comparison of Figures 2 and 3 reveals a distinct correlation between the intensity of illumination and the length

of time required to reverse the phases of an established rhythm. Thus it is seen from Figure 2 that with illumination of 150 foot-candle intensity the reversal occurs in the first day; from Figure 3, that with illumination of 80 foot-candles the reversal occurs in the third day; and, again from Figure 2, that with illumination of about 40 foot-candles reversal occurs on the fourth day. It may be noted that the last case involves a transition from a recently induced reversed rhythm back to a normal one so far as the actual time-relationships of its phases are concerned, and reversal might, for this reason, be expected to be more easily achieved here than in the other two cases. The latter, however, is obviously not the case.

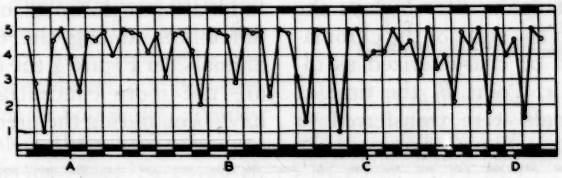

FIG. 3.—*Uca* in constant darkness were, beginning at *A*, illuminated at night (7:00 P.M. to 7:00 A.M.) and kept in darkness during daytime (7:00 A.M. to 7:00 P.M.). Beginning at *B*, they were kept in constant darkness up to time *C*, when they were exposed to 6 hours of illumination alternating with 6 hours of darkness. At *D*, following a final 6-hour period of illumination, they were placed in constant darkness.

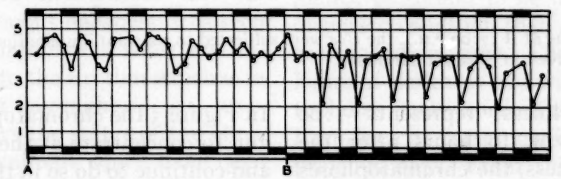

FIG. 4.—Beginning at *A*, *Uca* were exposed to continuous illumination; at *B* (7:00 A.M.) they were transferred to constant darkness.

Figure 4 shows the effect upon the chromatophores of constant illumination (beginning at *A*) followed by continuous darkness (beginning at *B*). It is seen that illumination at an intensity of 80 foot-candles immediately results in a marked decrease in amplitude of the rhythm. Nevertheless, it is possible to distinguish the rhythm clearly for the first 2 days. After this the rhythmical character of the changes in the state of the chromatophores becomes gradually less evident until, by the sixth day, such changes as are recorded appear to be arrhythmical. The time at which the animals were changed from light to darkness (point *B*) is 7:00 A.M. The first time that a marked degree of concentration of the pigment is recorded is 24 hours later, at 7:00 A.M. From this point on, a distinct 24-hour rhythm is observed, with the most concentrated condition of the pigment occurring at 7:00 A.M. instead of the original 1:00 A.M. The amplitude of the rhythm never becomes so great as that usually observed under conditions of constant darkness for animals otherwise untreated. A comparable shift in rhythm of retinal-pigment migration was observed by Smith (1948) following similar treatment.

Figure 5 presents a record of chromatophores under constant illumination, beginning at *A*, followed by constant darkness, beginning at *B*. The illumination was of about 80 foot-candle intensity. The results of constant illumination here are seen to be similar to those shown for similar conditions in Figure 4. The amplitude of the rhythm appears to be somewhat greater in the first few days (in Fig. 5), but again after 6 days the small changes in the state of the chromatophores cannot be characterized as diurnally rhythmic. Point *B*, the time at which the animals were transferred to continuous darkness. At 1:00 A.M. (6 hours after the change to darkness occurred) the chromatophores are seen to be almost completely concentrated, and, from here on, a 24-hour rhythm, in phase with solar day-night, is observed.

If one now examines those parts of Figures 4, 5, and 6 that show the conditions of the chromatophores for the first 12 hours following the change from illumination to darkness, one finds distinct differences among the three groups of animals. In Figure 4 the chromatophores are not more concentrated than they have been during the preceding 24 hours.

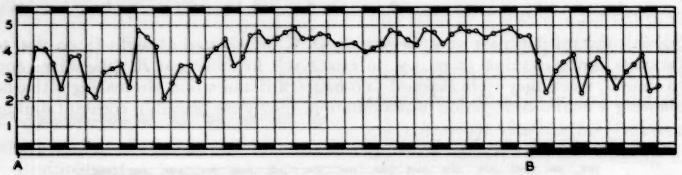

FIG. 5.—Beginning at *A* (7:00 P.M.), the *Uca* were subjected to constant illumination (80 foot-candles); at *B* they were removed to constant darkness.

continuous darkness, represents 1:00 P.M. At 7:00 P.M. (6 hours after the change to darkness) the chromatophores are found to be more concentrated than at any time in the preceding several days. At 1:00 A.M. they are still more concentrated, and, from this time on, a 24-hour rhythm, in phase with solar day-night, is observed.

Figure 6 shows the state of the chromatophores of a third group of animals exposed to constant illumination, beginning at *A*. The results are similar to those seen in Figures 4 and 5, showing, first, a decrease in amplitude and then a gradual inhibition of rhythmicity in the changes of the chromatophores. The time required for inhibition is approximately the same as in the two preceding experiments, and the intensity of illumination is the same—80 foot-candles. At *B* (7:00 P.M.) the animals were transferred to

In Figure 5 the chromatophores have begun to concentrate at the end of 6 hours and continue to do so in the next 6 hours. Finally, in Figure 6 it is seen that almost complete concentration of the chromatophores has occurred within 6 hours and that by the end of 12 hours a considerable degree of dispersion has occurred. The external conditions have been the same in all three cases—namely, a change from light to darkness following prolonged illumination at 80 foot-candle intensity. The only difference has been the time of day at which the change was made. It would seem, therefore, that, even though the state of the chromatophores in the three groups of animals gave no indication of internal differences among the animals, such physiological differences must have existed to account for the varying responses obtained.

Figure 7 shows the states of the chro-

matophores under the following conditions: a preliminary period of continuous darkness, alternate 6-hour periods of illumination and darkness beginning at *A*, and, finally, a period of continuous darkness beginning at *B*. The initial period of illumination (80 foot-candles) occurred from 7:00 P.M. to 1:00 A.M., and the results are seen immediately. The chromatophores are in a more dispersed condition at 1:00 A.M. than would be expected under normal conditions. The next point on the graph (7:00 A.M.) represents the end of a period of darkness, and it is seen that the chromatophores are slightly more concentrated than they were at 1:00 A.M. From this time to point *B*, when the animals are returned to continuous darkness, the chromatophores exhibit a 24-hour rhythm which is approximately 6 hours out of phase with solar day-night. When the animals are returned to continuous darkness, it is seen that the chromatophores show an immediate return to the rhythm which is in phase with solar day-night.

Figure 8 shows the states of the chromatophores of animals which have been exposed to illumination at night and darkness in the daytime for a period of 1 week and were then placed in constant

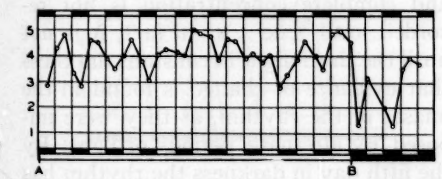

FIG. 6.—Beginning at *A*, *Uca* were exposed to continuous illumination; at *B* (7:00 P.M.) they were transferred to constant darkness.

darkness. Point *A* represents the first time that the condition of the chromatophores was recorded following the transfer to constant darkness. At point *B* alternate 6-hour periods of illumination and darkness were initiated. The illumination was of 80 foot-candle intensity. These periods were continued to point *C*, at which time the animals were again placed in continuous darkness. Between *A* and *B* a 24-hour rhythm of good amplitude, but with the phases the reverse of

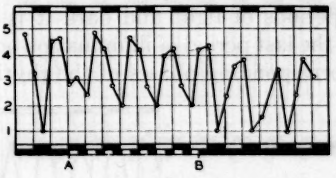

FIG. 7.—*Uca* in constant darkness were, beginning at *A*, subjected to alternating 6-hour periods of light and of darkness. At *B* (1:00 P.M.), following a period of illumination they were placed in constant darkness.

those found under normal conditions, is exhibited. At the end of the first 6-hour period of illumination the chromatophores are found to be in a much more dispersed condition than at any previous corresponding time. The succeeding 6 hours of darkness end with the chromatophores more concentrated than they have been at corresponding times of the previous days. From this time on, the most concentrated condition of the chromatophores occurs 6 hours later than it had in the previously established reversed rhythm. When the animals are returned to constant darkness, at point *C*, it is seen that there is an immediate return to the reversed rhythm, with the chromatophores being most concentrated at 1:00 P.M.

A comparison of Figures 7 and 8 on the basis of the phases of the rhythm at which certain events occur shows a number of similarities. Thus it is seen that the initial 6-hour period of illumination occurred in both cases at that phase of the rhythm in which concentration of the chromatophores would be expected to occur (point *A* in Fig. 7; point *B* in Fig. 8). In both cases not only did concentra-

tion not occur but a certain degree of dispersion is indicated. In both cases, too, the ensuing period of darkness is accompanied by concentration of the chromatophores at a time when, on previous days, dispersion has been found to occur. Again at the end of exposure to alternating periods of light and dark it is seen that the last period of illumination occurs at corresponding phases of the rhythms.

Figure 9 represents the condition of the chromatophores of animals exposed to alternating 6-hour periods of light and darkness, beginning at *A*. At *B* the animals were transferred to continuous darkness. The results of exposure to alternating periods of light and darkness are similar to those seen in Figures 7 and 8, in that the time of greatest concentration of the chromatophores is shifted and

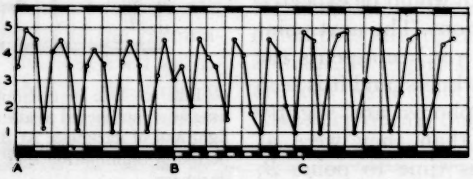

FIG. 8.—*Uca* whose diurnal rhythm had previously been reversed by illumination by night and darkness by day were, beginning at *A*, kept in constant darkness. Beginning at *B*, the animals were exposed to alternating 6-hour periods of light and of darkness. At *C* (1:00 A.M.), following a period of illumination, they were returned to constant darkness.

In both cases the first 6 hours of darkness (following *B* in Fig. 7, *C* in Fig. 8) are accompanied by little or no concentration of the chromatophores, while at the end of 12 hours the chromatophores of all the animals are found in stage 1.

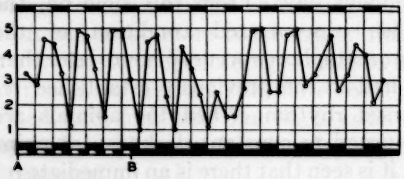

FIG. 9—Beginning at *A*, *Uca* were exposed to alternating 6-hour periods of light and of darkness. Beginning at *B* (1:00 A.M.), following a period of illumination they were kept in constant darkness.

There is, therefore, no suggestion in the results of these experiments that the observed rhythm in those animals which had been exposed to reversed illumination is dependent on anything less basic than is the rhythm of animals not so treated. The physiological condition, as judged by their response to similar treatment, is the same.

occurs 6 hours later than is normally found. In the first 6 hours of darkness following point *B*, it is seen that the chromatophores have become completely concentrated. One might, perhaps, expect that at the following 1:00 A.M. the chromatophores would be completely concentrated, since there has been no intervening period of illumination to cause dispersal. Such, however, is not the case, and complete concentration is not recorded until 7:00 A.M. In fact, it is not until the third night in constant darkness that evidence of change is found in the phases of the rhythm, as they were imposed by alternate periods of light. By the fifth day in darkness the rhythm has become in phase with solar day-night.

It will be recalled that in Figure 3 data were recorded from an experiment in which animals were subjected to reversed illumination until reversal of the chromatophore rhythm was observed and then were exposed to alternating 6-hour periods of light and dark. A comparison of Figures 3 (from point *C*) and 9 shows

that illumination was begun at corresponding phases of the rhythms in both cases. The last period of illumination likewise occurred at comparable periods. It is further seen that the first 6 hours of darkness (point *D* in Fig. 3; point *B* in Fig. 9) ends with the chromatophores showing complete or almost complete concentration. The second 6 hours of darkness is accompanied, in both cases, by practically complete dispersal. It therefore seems that these two groups of animals are responding to external conditions in a manner qualitatively the same at corresponding phases of their rhythms.

Figure 10 represents the condition of the chromatophores of animals kept in darkness except for 1-hour periods as indicated by *A*, when they were exposed to illumination of 80 foot-candle intensity. These periods of illumination occurred from 7:00 A.M. to 8:00 A.M. The only difference seen between the states of the chromatophores here and that found for the control group (Fig. 1) is a small difference in amplitude.

Figure 11 shows the state of the chromatophores of animals kept in darkness except for those periods labeled. At points labeled *A* the animals were illumi-

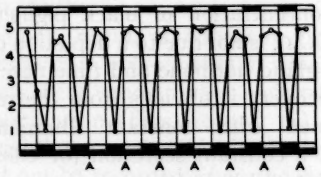

FIG. 10.—*Uca*, otherwise in constant darkness, were subjected to 1-hour periods of illumination at points *A* (7:00 A.M. to 8:00 A.M.).

nated for periods of 1 hour at an intensity of 80 foot-candles (7:00 P.M. to 8:00 P.M.). Point *B* marks the beginning of a 6-hour period of illumination; *C*, the beginning of a 4-hour period; and *D*, a 3-hour period. Following the 6-hour period of illumination there is observed a marked distortion of the normally observed rhythmical pattern. The distortions at *C* and *D* were progressively less.

It is impossible, however, to explain the observed distortions in terms of a

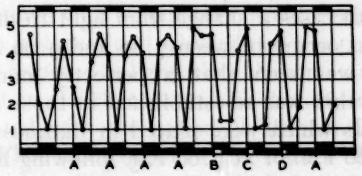

FIG. 11.—*Uca*, otherwise in constant darkness, were subjected at points *A* to a 1-hour period of illumination; at point *B* to a 6-hour period; at point *C* to a 4-hour period; and at point *D* to a 3-hour period.

specific mechanism. Thus, at the end of the 6-hour period of illumination beginning at *B*, the chromatophores are almost completely concentrated. This is in contrast with all the previous experiments, in which it was found without exception that 6 hours of illumination at the same phase of the rhythm resulted in the chromatophores being no more concentrated than in stage 3. It is unfortunate that points *C* and *D* were periods of illumination longer than 1 hour, since previous experiments lead one to suspect that the return to normal phase would have been similarly gradual, even had the crabs been placed in constant darkness.

DISCUSSION

The results which have been described may be classified, for purposes of discussion, into three general groups: (1) those in which there was effected a shift in the rhythm and this shift was persistent; (2) those in which there was effected a shift in the observed rhythm which disappeared following the removal of the stimulus; and (3) those in which the imposed conditions had no effect on the observed rhythm.

In group 1 we find the results of reversed illumination (Figs. 2 and 3) and the results of placing the animals in continuous darkness at 7:00 A.M., following inhibition by constant illumination (Fig. 4). If we now look for a factor common to these two experiments, we see that in both cases a change from light to darkness occurs between 7:00 and 9:00 A.M. It was seen that a perfectly normal rhythm was re-established in those animals which were placed in darkness at 1:00 P.M. or at 7:00 P.M. following light inhibition of the rhythm (Figs. 5, 6, 7). This difference in susceptibility to the stimulation involved in the change from light to darkness implies a rhythmicity which has persisted during the continuous illumination, even though the condition of the chromatophores no longer reflects the rhythmicity after a certain time. The mechanism suggested by Welsh (1941) for the control of retinal pigment migration affords a possible basis for such a persistent rhythmicity. Welsh postulated that tonic impulses from the fourth optic ganglion or from the "brain" inhibit release of the hormone from the sinus gland; stimulation of the eye by light causes reflex inhibition of the inhibitory centers and results in secretion by the sinus gland. If it is assumed that light acts not by inhibiting the center but by preventing transmission of inhibitory impulses, it is possible to explain constant secretion by the sinus gland while the inhibitory center is still undergoing cyclic activity. Then it is necessary only to assume that a period including the time 7:00 to 9:00 A.M. corresponds to a point in the phase of the "inhibitory rhythm" at which the basic rhythmical process can be stimulated by a change from light to dark.

A mechanism to explain the fact that in one case (placing in darkness at 7:00 A.M.) the persistent rhythm is 6 hours out of phase with the normal, while in the other case (reversed illumination) it is 12 hours out of phase, is not immediately obvious. It is true that in the first case (6 hours out of phase) the change was brought about by a single stimulus—the change from continuous illumination to continuous darkness. The rhythm which is 12 hours out of phase was the result of stimulation by light during the time when the chromatophores are normally concentrated, followed by the change from light to darkness at the "sensitive period" of the inhibitory rhythm.

Consideration of the results in group 2 (Figs. 3, 7, 8, and 9) emphasizes the importance of stimulation of the postulated inhibitory rhythm. It is seen that in no case in these experiments has the change from light to darkness occurred at the sensitive period of inhibition. In this group the results depend upon stimulation by light at a time when the chromatophores would normally be concentrating. Under these conditions the chromatophores tend to remain dispersed when they would normally be concentrated, and the whole observed rhythm is shifted by an amount corresponding to the enforced dispersal period. That this shift in rhythm, now involving greatest concentration of black pigment at 7:00 A.M., is not due to a depletion of the sinus-gland hormone and time necessary for recovery is obvious from the results obtained with constant illumination. There a constant secretion (or constant high blood level of hormone) was maintained for several days (Figs. 5 and 6). That a basic rhythm which results in a rhythmical inhibition of the sinus gland has retained its normal phase is suggested by the return to normal after removal of the stimulus. In group 2, as in group 1, two types of results are observed which are not easily explainable. In this case it is the difference in time required for a re-

turn to the earlier phase of the rhythm. The only obvious difference in treatment between the two kinds of experiments involves the time of the last period of illumination. When the last period of illumination is that from 7:00 A.M. to 1:00 P.M. or a corresponding time in a previously reversed rhythm (7:00 P.M. to 1:00 A.M.), the return to the earlier rhythm is immediate. On the other hand, when the last period of illumination is from 7:00 P.M. to 1:00 A.M. or a corresponding time in a previously reversed rhythm (7:00 A.M. to 1:00 P.M.), the return to the earlier rhythm occurs gradually over several days.

The immediate return permits the interpretation that illumination at the 7:00 P.M. to 1:00 A.M. phase of normal rhythm extends the period of secretion but has no effect on the underlying rhythmicity. If, however, this were a complete description of the process involved, one would expect the return to normal to be delayed only 1 day when the last period of illumination was from 7:00 P.M. to 1:00 A.M. followed by continuous darkness. In the experiments performed under these conditions, however, it was found that a period of about 5 days was necessary for a gradual return to the earlier rhythm.

The only results to appear in group 3 are those from experiments in which the animals were exposed to 1-hour periods of illumination (Figs. 10 and 11). It is realized that a 1-hour shift in the rhythm would not be recorded when the state of the chromatophores was determined at 6-hour intervals. The results of exposing the animals to light from 7:00 A.M. to 8:00 A.M. suggest that a longer period of light adaptation is necessary in order to elicit a response to the change from light to darkness. It will be recalled that reversal was obtained not only when the illumination period was 7:00 P.M. to 7:00 A.M. but also when the period was from 9:00 P.M. to 9:00 A.M. and thus under conditions such that the change to darkness occurred at a later time.

The lack of observed effect of illumination from 7:00 P.M. to 8:00 P.M. is not unexpected. If, as seems likely, the effect obtained with 6-hour periods of illumination is dependent primarily on a superficial response to light, then the amount of shift in the rhythm will be directly correlated with the period of illumination.

When one considers the results illustrated in Figure 9, in which a rhythm caused to become 6 hours out of phase with normal, reverts back to the normal phase gradually over several days in constant darkness, one is led to the conclusion that two rhythmical centers must be involved. In this experiment a single 6-hour period of illumination from physiological 7:00 P.M. to 1:00 A.M. of the rhythm was apparently sufficient to induce the 6-hour change in phase of one of the two centers, since the next 6-hour period of illumination in the 6-hour alternating periods of light and dark apparently restored the cycle to the normal phase (Figs. 7 and 8). This stimulus, which induced the 6-hour change in one center, apparently caused no alteration in a second center, which possessed the ability to restore gradually the altered cycle to its original phase.

All the results reported in this paper appear explicable in terms of a hypothesis in which two centers of rhythmicity are operating and in which the rhythmicity of one center is capable of being modified by the other, but not the reverse. Figure 12 is a diagrammatic representation of such a hypothesis and of some of the apparent characteristics of its activity. Center 1 is considered to be the more basic center and to possess an inherent 24-hour rhythmicity. Center 1 can control center 2, which might be

called the "color-change center." Center 2 appears to be more closely associated with the controlling mechanism for the chromatophores and serves as the only route through which center 1 can influence color changes. Center 2 may also show a rhythmical activity, but one which is less persistent than that of center 1.

Center 2 is postulated, on the basis of the inhibition of the rhythmicity of color change in constant illumination (Figs. 4, 5, and 6), to lose its rhythmicity gradually following an inhibition of a rhythmical inhibition from center 1; center 2 is rendered rhythmic again immediately upon removal to constant darkness by activity of center 1, whose rhythmicity persisted during the period of constant illumination.

Just as with center 2, so center 1 appears capable of having its rhythm altered by 6 hours by a single change in illumination during a "sensitive" period. Since both centers 1 and 2 characteristically appear to be altered abruptly by single light changes or illumination periods, the "two-center" hypothesis also explains very nicely the behavior during the reversal of the rhythm by reversed illumination following *A* in Figure 3 and *C* in Figure 2. Here it is postulated that a single 12-hour period of illumination at *A* and following *C*, respectively, caused center 1 to become 12 hours out of phase with its original rhythm while not altering the rhythm of center 2. As was true when center 2 was 6 hours out of phase with center 1 (Fig. 9), so here, too, when it is 12 hours out of phase, several days

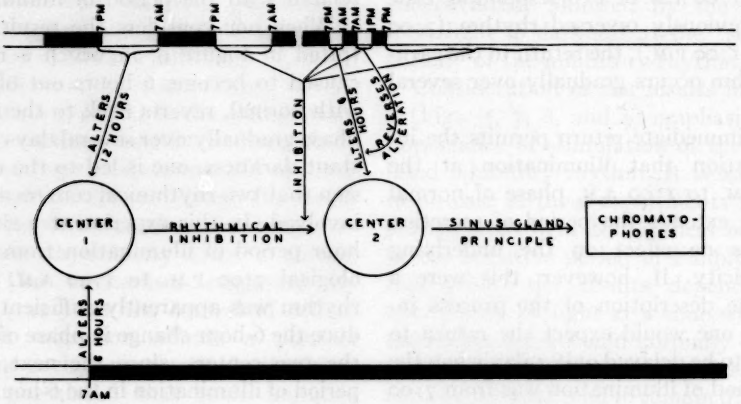

FIG. 12.—Diagrammatic representation of a hypothesis to account for the observed characteristics of modifiability of the daily rhythmicity in *Uca*.

are required to enable center 1 to bring center 2 into phase with it. On the basis of this hypothesis the gradual change to the reversed phase should have continued though the animals were returned to constant darkness after the first 12-hour period of reversed illumination. If this did not occur, then there would still be the possibility that center 2 could influence center 1, at least under some circumstances. This last type of experiment should be performed.

Lending support to the hypothesis that reversed illumination alters center 1, which then gradually alters center 2 over a period of days, are the general characteristics of the transitional period. It should be recalled that the rhythms of

the two centers, according to this concept, would be in exactly opposite phases. It would be expected that one would gradually neutralize or inhibit the other, and, after inhibiting it, the more persistent would contribute its own phase characteristics to the other. This appears to be precisely what has occurred, with center 2 inactive and the chromatophore pigment widely dispersed (recall, also, the broad pigment dispersal accompanying inhibition of the rhythm of center 2 by constant illumination), after about 2 days, in Figure 3. There is a sharp transition to the phase of the new rhythm immediately afterward. In contrast with this, when center 2 was only 6 hours out of phase with center 1 (Fig. 9), and hence there was not complete antagonism existing between the centers, the rhythm gradually shifted through the 6-hour interval to the phase of center 1 without such a transitional inhibition; this also is just as might be anticipated.

The foregoing hypothesis is an interpretation of the observations in terms of two rhythmical centers, each capable of being altered in the character of its rhythm by illumination changes independently of the other, as a result of differences either in periods of sensitivity to light stimuli or in the general patterns of their adequate stimuli. Upon the basis of this hypothesis, it is assumed that the alteration in the rhythm by 6 hours as a result of chilling the animals for 6 hours (Brown and Webb, 1948) must have been due to a temporary inhibition of center 1; center 2 could similarly have been temporarily inhibited or could have had its own rhythm stopped, to be started anew only by, and in phase with, center 1.

SUMMARY

1. It has been shown that the diurnal rhythm of the chromatophores of *Uca* is inhibited by constant illumination.

2. A basic physiological rhythm persists in the animals even when the chromatophoric expression of the rhythm has been thus inhibited.

3. It is possible to reverse the phases of the diurnal rhythm of the chromatophores by illuminating the animals by night and keeping them in the dark by day.

4. Under conditions of reversed illumination there is no evidence that the basic physiological rhythm does not become completely in phase with the imposed rhythm.

5. Exposure of the animals to 6-hour periods of illumination alternating with 6 hours of darkness results in the rhythm of the chromatophores becoming 6 hours out of phase with a previously observed rhythm.

6. Such a rhythm, 6 hours out of phase, may persist for several days in constant darkness and then gradually return to a previously established rhythm if the last period of illumination occurred when the animals were entering the night phase; but it shows no persistence whatsoever when the last period of illumination occurred when the animals were entering the day phase.

7. All those cases of alteration in rhythmicity in which a clear answer is possible suggest that, when a rhythm is altered, it is altered abruptly by a single light change or by a single period of illumination.

8. An analysis of all the data has led to the adoption of a hypothesis of two centers of rhythmicity within the animal, each one capable of having its rhythm altered independently of the other, and with one of the centers—that one having a more persistent rhythmicity—influencing the other.

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MATING BEHAVIOR OF TWO LIVE-BEARING FISH, XIPHOPHORUS HELLERII AND PLATYPOECILUS MACULATUS

HAROLD SCHLOSBERG,[1] MARIE CASTALDI DUNCAN[2], AND BETTY HORENSTEIN DAITCH[1]

THIS paper reports a series of studies of the mating behavior of two live-bearing fish, *Platypoecilus maculatus* and *Xiphophorus hellerii*. They are two of the most common "tropical" fish and are commonly known as "Platies" and "Swordtails," respectively. Their popularity in home aquariums can be attributed largely to the ease with which they can be mated and reared. Unlike most fish, the females of this group (Poeciliidae) retain the young until the yolk-sacs have been absorbed, so that the fry are able to survive without special attention. Furthermore, the litters or broods are of a convenient size, ranging around 20–40 at a time, delivered approximately monthly.

These same characteristics make the two species convenient ones for certain research purposes. For example, Gordon (1947) and Bellamy (1924) have made extensive studies of certain genetic factors in both species. They furnish especially interesting material for the geneticist, because they cross-breed, yielding fertile intergeneric hybrids.

Under normal aquarium conditions the fish are almost constantly engaged in one of three types of activity: eating, mating, or practicing dominance and submission. The latter behavior has recommended the Platy to those interested in social organization in animals. Thus Braddock (1945) has studied the hierarchical structure of new and of established groups of these fish.

One of the most characteristic things about these fish is the persistent attempts of the males to mate. Since the fish are live-bearers, it is necessary for the male to fertilize the ova within the female's body. The intromittent organ is developed as a modification of the anal fin into a troughlike structure. This gonopodium is normally carried pointing

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to the rear, close to the body; during mating, it is pulled forward and to one side, as the male approaches the female from below and behind and thrusts at her vent. When successful contact is made, a sticky capsule of spermatozoa is inserted into the female genital passage. In the folds of the lining of the oviduct the spermatozoa may live for months, fertilizing as many as 5 or 6 litters (Henn, 1916; Turner, 1937). Thus all healthy females in a tank with one or more males are usually pregnant; nevertheless, the males constantly dart around the females, "courting," or jockeying for a position from which to start a thrust.

The factors which control mating behavior in these and related species have already received some attention. Noble and his associates (1938) believed that the sexes recognize each other through visual means. It should be pointed out that there is marked sexual dimorphism. The female changes very little at puberty. Her chief sign of sexual maturity is the development of a black "puberty spot" in the region of the pelvic fins. This spot results from pigmentation of the peritoneal lining of the body cavity enveloping the ovary. A healthy female continues growth after puberty and also becomes quite plump. On the other hand, the male is likely to cease growth at puberty, remaining slender, and averages about two-thirds the length of the female. Of course, puberty in the male is marked by the development of the intromittent organ. In *X. hellerii* there is another striking change—the male develops the swordtail from which he gets his name. The sword is a thickening and extension of the ventral margin of the caudal fin into a long filament. This sword may be as long as the rest of the body. It is further accentuated by a line of black pigment and often by a continuation of either the green color of the body or the reddish hue of the lateral stripe. The function of this sword has interested a large number of people, beginning with Darwin (1871), who considered it to be a form of adornment.

A complete understanding of mating behavior must go far beyond determining the stimuli which control it, for there are neural and hormonal factors to be considered. There has been relatively little work on the neural centers involved. Noble and Borne (1941) have shown that mating behavior in *X. hellerii* survives total ablation of the forebrain. But in the Cichlids, egg-laying fish which show elaborate courting and parental behavior, the forebrain seems to be more important (Noble, 1939; Noble and Borne, 1941). As for hormonal factors, it is sufficient to point out here that the same glandular secretions function similarly throughout the vertebrates. The present status of our knowledge on the topic has been adequately summarized by Beach (1948). *Platypoecilus maculatus* and *X. hellerii*, as well as the guppy (*Lebistes reticulatus*) have been widely used for such research (Eversolè, 1941; Grobstein, 1940; Noble and Borne, 1940; Sangster, 1948). Their marked sexual dimorphism offers opportunity for anatomical, as well as behavioral, changes. Thus Eversole has shown that pregnenolone or testosterone will cause female guppies to develop male behavior and male secondary characteristics.

But, if one is to investigate the effects of neural and hormonal factors on sexual behavior in a given species, it would seem important to have an adequate description of the normal sexual behavior of that species. The studies reported in this paper represent a step in that direction. More specifically, they include a careful analysis of mating behavior in *X. hellerii*, and a less complete study of *P. maculatus*.

I. The Fish and Their Maintenance

The original stock, obtained from the Genetics Laboratory of the New York Aquarium, New York Zoological Society, was third- to fourth-generation offspring of wild fish which Dr. Myron Gordon had collected in Mexico. There are many strains of these fish available through dealers, but, since even the two different genera yield fertile hybrids when bred in captivity, one cannot be sure of the genetic background of the commercial strains.

Most of the stock tanks were standard 5-gallon rectangular ones, covered with glass. Temperature was maintained at 75°–80° F., either by control of room temperature or by immersion heaters and a thermoregulator. Each tank had an inch of sand on the bottom and was planted with *Vallisneria* or *Nitella*. The latter plant is especially useful for protecting newborn young from their predatory parents. It also harbors microörganisms that serve as food. During the winter months artificial light, controlled by an electric clock, was used for 12 hours daily. This was necessary for the growth of plants; it also seemed advisable for its effect on sexual behavior (Hoover and Hubbard, 1937; Turner, 1937). Snails (either *Planorbus* or *Physa*) served as scavengers. A few poorly placed tanks suffered from too little light or excess heat, so that the plants gradually died and the water became dirty; fish from these tanks were not used in the experiments. The remaining tanks were well balanced and remained clear. Aeration was maintained in most tanks as a safety measure.

A pregnant female was placed in a well-planted tank. As soon as the young were born, she was removed, to prevent her from eating the young. Within a month, the young were separated, 6 to a tank, to encourage growth. In 3–12 months the males developed the modified anal fin that serves as a copulatory organ. At this time they were transferred to another tank and rearranged as necessary to keep 3–7 fish of the same sex in each tank. All fish were fed sparingly, twice daily. The morning feeding was ground and sifted Purina Dog Chow, while the second feeding varied between a commercial food ("Fish Manna") and ground salmon eggs. During the most careful observation a mixture of liver and Pablum was used to supplement the diet. Under these conditions the fish usually stayed in good health for 2 or 3 years, which is apparently their normal life-span.

The fish were sometimes observed in their normal tanks, either segregated by sex or mixed for breeding. But controlled observations were made in special tanks, free of vegetation and snails. These tanks varied from small ($2\frac{1}{4}$ gal.) to large (15 gal.). The fish were isolated before and between observation sessions, either in gallon jars or in 15-gallon tanks divided into eight compartments by plastic screening. They were normally placed in the observation tank at the beginning of the observation period and removed at the end. The usual duration of each session was 40 minutes. In certain cases a pair of fish was left in the observation tank between sessions, but separated by a glass partition. This served as a check on the effects of moving the fish from the home tank to a strange one.

II. Mating Behavior of *Xiphophorus*

A. Qualitative observations

These observations were made under varied conditions, in an attempt to get a rough idea of the mating behavior. In some cases the fish were observed in bal-

anced aquariums with several fish of one or of both sexes present. At other times 2 fish were placed in a clean 5-gallon tank. The following responses were observed.

1. MALE RESPONSES

a) Arcing.—This pattern has been observed by everyone who has had Swordtails. In its most typical form the male stands poised in front of the female, fins erect and quivering, with the body often rigidly bent in a sigmoid pattern. He is at right angles to her body axis, an inch or two in front of her mouth. If she does not move, the male usually curves the tail toward her and swims backward. This often causes the tail to strike the female's flank and has led some observers (Kosswig, 1936; Van Oordt, 1925) to assert that the function of the sword is the stimulation of the genital papilla or flank of the female. However, a somewhat rigid prolongation of the tail fin would seem to be a rather inaccurate device for such purposes.

If the female responds to the stationary phase of arcing by turning and swimming away, the male swims rapidly as if attempting to maintain his relative position in front of her. This often involves rapid and jerky dashes, alternating between forward and backward movements. The presence of the sword and the unusual sight of a fish flashing backward the full length of the tank has attracted undue attention to this behavior. It is easy enough to assume that the male is trying to touch the female with the sword or to intimidate her or to corner her (Noble, 1938). The present writers are more inclined to believe that arcing, in front of either a stationary or a moving female, is more in the nature of a visual display. It will be pointed out later that the same behavior is found in the Platy, but no one has commented on it because the swordless Platy is less impressive when swimming backward.

The arcing response is also made to other males. In fact, it can be seen most frequently when 4 or 5 males are kept in a single tank. It goes on intermittently throughout the day for week after week. Removal of the sword does not stop the response. The most plausible hypothesis would seem to be that arcing is a form of display or challenge, which may eventually lead to mating or to establishment of dominance-submissive relationships.

b) Nipping.—This pattern occurs with a high frequency during the preliminary phase of mating. In its most typical form the male starts from one side and slightly below the female and lunges at her flank, often in the region of the genital papilla. He usually slows down before contact and rarely moves the female by impact. Frequently, there is no actual impact, and the nip might be called a "feint." Further, the nip may be delivered to any part of the body.

Although the nip is frequent in courting, there is no reason to believe that it is specific to mating behavior. It is perhaps the most general response that the fish can make and appears in picking up and investigating particles on the bottom of the tank, in "passes" at snails, and in aggressive behavior toward other fish. Females show the same response (see below). It varies greatly in intensity and velocity from situation to situation. Whatever effectiveness it has as a weapon must come from the force of impact, for this family of fish has small mouths and teeth. The function of the nip in courting is probably quite similar to that of arcing: it stimulates reactions in an inactive female. It is often alternated with arcing or with other forms of body contact, such as swimming under the female so that the dorsal fin strikes her body or vent.

c) Isolated flexion of the gonopodium.—This reaction is the most frequent male response. The modified anal fin is reversed from its normal position under the belly and is swung forward and down. Within a second it usually returns to the resting position. It may be a localized response, or it may be accompanied by a convulsive jerk of the body. It is not directed toward the female but seems to be a preparatory response.

Henn (1916) pointed out that it is uncertain how the spermatophore gets to the end of the intromittent organ in this group of fish. The modified anal fin is more like a trough than a tube and has no obvious mechanism for moving the sperm. Collier (1936) reported that in a related fish, *Gambusia*, the ligaments which control the gonopodium are also associated with a saclike ejaculatory sphere, located at the base of the gonopodium. He said: "These ligaments are probably the means by which the ejaculatory sphere is contracted so as to eject the spermatophores, when the anal fin is reversed. The reversal of the anal fin is commonly observed to precede coitus in various members of Poeciliidae." He further assumed that the spermatophore is held by the hooks which are at the tip of the gonopodium, to be transferred to the female on contact.

d) Thrust.—This is the actual mating response; if successful, it brings the gonopodium into contact with the female genital papillae. It may begin from a foot away with little preliminary courting, but more frequently it comes as the culmination of a long sequence, including jockeying for position, or arcing.

The normal position for the start of a thrust is just below and an inch or two behind the female. The thrust can usually be anticipated by observing an increased activity of the pectoral fins. These fins are in constant motion, even when the fish is stationary, for they counteract the surge of water through the gills. Hence their increased activity may be comparable to the preparatory change in breathing which precedes activity in higher animals. In executing the thrust, the fish spurts forward and upward, simultaneously swinging the intromittent organ forward and toward the female's vent. As he approaches her flank, he tilts and curves his body to bring the intromittent organ toward her vent. In the extremely infrequent cases in which the thrust hits the vent, the two fish stay together for a few seconds and then separate. But the intromittent organ almost invariably glances off the abdomen or flank, and the male slides past the female. They may then repeat the process, in a *courting run* (see below) or they may not.

2. FEMALE RESPONSES

a) Posing.—This response is probably somewhat analogous to arcing in the male. In the presence of an inactive male the female may swim to a position in front of him. If he does not respond, she may swim off and repeat the posing a few minutes later. If he responds by nipping her genital region, the female usually flexes her pelvic fins. After several repetitions of this sequence, the male is likely to make a thrust. In addition to flexion of the pelvic fins, the female may also flutter the caudal fin vigorously during a pose. This may well be an attention-getting device, since there is evidence that movement is an important aspect of visual stimulation of the mating in this species (Noble, 1938).

b) Nipping.—The female will occasionally nip an inactive male; it is hard to tell whether this is sexual or aggressive behavior. It is probably sexual when it is directed toward the male's gonopodium. But nipping is clearly aggressive behav-

ior when the female responds to a nip by a vigorous return nip. When this happens, either the male withdraws, or the two fish chase each other around in circles, each nipping at the other's tail.

3. JOINT ACTIVITY

a) *The courting run.*—When a male makes a thrust, the female may make no obvious response, or she may dart off to one side. But if she moves forward slightly, flutters the caudal fin, and flexes the pelvic fins, the male usually repeats the thrust, and the cycle is repeated from two to twenty times in as many seconds. This sequence is called a "courting run." To the casual observer it appears that the female is attempting to escape from the male, but actually the run is a nicely co-ordinated sequence. After the male thrust, the female darts forward an inch or two, stops, unfurls her pelvic fins, and remains stationary until the next thrust. In the meantime, the male has backed up enough to be in position to start another thrust. The run is usually broken off by the female after it has carried the pair to the end of the tank, but it may continue for another trip across the tank, if the turn has not been too sharp. Runs may be repeated a half-dozen or more times in an hour.

From this discussion it is apparent that the male thrust is quite inaccurate. Only one in several hundred yields a contact that lasts as long as a second; the rest must either be "misses" or must be blocked by the female's ventral fins. The detailed action of gonopodium and of the female's fins is extremely difficult to observe, for the action is quite rapid.[3] But it is obvious that ventral and anal fins must play an important part in the act; for they could either guide or obstruct the intromittent organ, depending on their precise position at the critical moment. It may be relevant to note that the male will often change sides once or more during a single courting run, suggesting that the female's fins are held in an asymmetrical fashion.

The courting run is usually initiated by the male, but in at least three cases it was clearly instigated by the female. In these instances the female obviously pushed against the side of the male, at the same time pushing him slightly forward. During these runs, the male did not change sides; they were also longer than the median for male-initiated runs, consisting of 12, 13, and 13 thrusts, respectively; the normal median is around 6.

B. RELATIVE INCIDENCE OF ELEMENTS OF COURTING BEHAVIOR IN *Xiphophorus*

1. METHOD

Six males and 8 females were selected for this study. When not under observation, they were kept in individual stalls of a battery tank. No rules were observed as to the particular stall in which an animal was placed, so that a specimen of one sex may or may not have been in sight of specimens of the opposite sex. Their usual diet was supplemented by a paste of Pablum and liver; they were never fed before an observation period.

Two animals were always observed while in a 5-gallon tank ($14 \times 8 \times 10$ inches) which was kept on a table in the center of the same room in which the fish lived. At this spot in the room the lighting was especially good, and there were no other fish closé enough to serve as distracting stimuli. The two junior authors made and recorded independent observations from their positions at opposite sides of the tank.

[3] Motion pictures were taken of the courting run, from the bottom as well as from the side and front, but they were disappointingly blurred at the critical times. The technical difficulties in the way of getting sharp pictures of such small, rapidly moving objects in a free field are great.

The data were collected during the hours between 10:00 A.M. and 12:00 M on weekdays. The procedure consisted of taking notes on the activity of 2 animals, 1 male and 1 female, which had been placed together in the observation tank. The temperature was recorded at the end of each observation period. The fish were then removed to the battery tank and replaced by a pair selected for study on the following day. A glass partition separated the pair when they were not under observation.

A rotation plan was devised so that each individual was observed with every other individual of the opposite sex, and no one specimen was ever observed more than twice during the same week. The plan was later abandoned when motion pictures were being taken, which necessitated the observation of the same pair for several consecutive days.

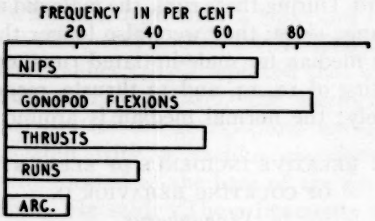

FIG. 1.—Frequency of occurrence of the various pattern types in the mating behavior of the male Swordtail. The length of the bars represents the percentage of sessions in which each of the responses was observed.

The histories of the individual subjects are unknown. Two of the fish, F-7 and F-8, were sexually immature, and F-8 was discarded after one observation, since she was much too young to stimulate any activity in the male. F-2 was also discarded early in the experiment because of a supposed impregnation, but she died before it could be proved. Because F-4 was inadvertently exposed to M-2 for an overnight period and was probably fertilized at the time, she was no longer used for experimental purposes. One male, M-5, died from no apparent cause late in the study, but, inasmuch as he had not been observed during the week previous to his death, his data were retained.

2. RESULTS

Each male, excepting M-3, attempted copulation with some female at one time or another. Each female, including immature F-7, served as an adequate stimulus for eliciting mating behavior in at least one male. M-2, who courted every female, was the only male to attempt copulation with F-5 and F-7.

The water temperatures recorded during the experiment ranged from 72°.5 to 88° F. with a median value of 79° F. Activity was noted at all temperatures, excepting at 72°.5 F., the lowest temperature recorded. Since the typical handbook on tropical fish (Innes, 1942) suggests that the optimal range for *X. hellerii* is 72°–80° F., the single observation at 72°.5 F. was discarded.

Figure 1 shows the relative incidence of the various elements of male courting behavior. The length of the bar indicates the percentage of observation periods in which each element was noted. The most frequent form of male activity—flexion of the gonopodium—occurred in over 80 per cent of the test periods. The male actually attempted copulation, by making a thrust at the genital papilla with his intromittent organ, in half the sessions. But the complete sequence—a courting run—was observed in only about one-third of the sessions. One of the possible preliminary phases of mating—nipping—occurred quite frequently, but the spectacular curving and backward swimming that we have called "arcing" was relatively infrequent.

Figure 1, in which all periods have been lumped together, shows that mating

does not always result if one places a pair of healthy fish in a tank. The failure is usually attributed to the male. In the present study, only 1 (M-3) of the 6 males failed to attempt copulation with at least 1 female. The other extreme is represented by M-2, who made thrusts at every female. A young male (M-6) reared in isolation, showed an unusually large number of brisk gonopodium flexions during the early part of his first mating period. Later in this session he made

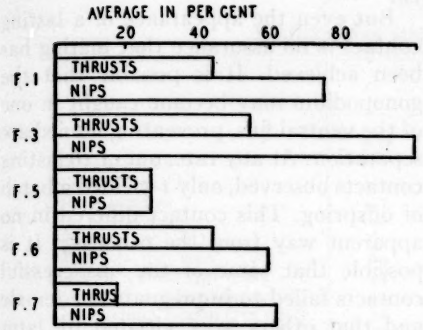

FIG. 2.—Stimulus value of each of 5 female Swordtails. The length of the bars represents the percentage of all observation periods in which each female elicited thrusts or nips from 1 of 4 males.

a number of inaccurate thrusts, which usually missed contact with the female. He failed to initiate a courting run until his second observation period, and he never showed the arcing pattern. This behavior is at least suggestive of an element of learning in the mating pattern (Noble, 1938).

But all the failure was not due to lethargy or inexperience on the part of the male. The females clearly varied in their ability to elicit sexual behavior. This can be seen from Figure 2, which presents the percentage of trials on which each of 5 females elicited mating behavior in 4 males (M-1 to M-4). F-3 apparently had a high stimulus value, for she elicited nipping in every session and thrusts in half of them. F-5 was the same age and appeared to be in similar health, but she evoked relatively little male activity. F-6 and F-1 were moderately effective, even though the latter was barely mature. On the other hand, F-7 was small and probably prepuberal; the fact that she called out frequent nipping and few thrusts is consistent with our earlier suggestion that the male nip is a challenge and that the resulting female response determines further male behavior.

The data of Figure 2 indicate that some characteristic of the female is an important determiner of male behavior. This characteristic may be her form or some chemical secretion, but it seems more probable that her behavior is the factor which determines her stimulus value for evoking the male mating response.

III. MATING BEHAVIOR OF *Platypoecilus*

A. METHOD

All the observations on the Platy were made by the senior author. In general, the methods were similar to those reported for the controlled study of the Swordtail (above). However, the sessions were distributed irregularly over three periods of 1 month each, 6 months between the periods. Incidentally, the similarity of the results from these three periods argues for the stability of the behavior.

The observations in the first two series (January, August, 1947) were made in $2\frac{1}{2}$-gallon tanks, whose dimensions ($12 \times 6\frac{1}{2} \times 9$ inches) were about four-fifths those used with the Swordtail. This change was made as a compensation for the smaller size of the Platy. But, when no courting runs were observed in these tanks, it seemed desirable to try larger ones, which would give the fish

more room. Hence, during the third series (February, 1948) the observations were made in a 12-gallon tank (20 × 12½ × 12½ inches). The results turned out to be independent of the tank size, so the two sets of observations will be considered together.

There were 48 observation sessions of 40 minutes each. Six males and 7 females were used during the first series, 2 males and 5 females in the second, and 2 males and 4 females in the third. Each fish was observed with at least 2 of the opposite sex. The time of day was not controlled.

B. RESULTS

The mating behavior of *P. maculatus* is similar in many ways to that of *X. hellerii*. The most clear-cut difference is the absence of the courting run in the Platy. When the male makes a thrust at her genital papilla, a co-operative female Platy may tip slightly and furl the anal and ventral fins, thus exposing her vent. She neither swims forward nor allows the impact to move her appreciably. When one realizes the ease with which a water-borne object can be moved by contact or by currents, it is obvious that her immobility is an active process. But, in spite of this immobility, the male Platy thrust is no more accurate than is that of the Swordtail. The gonopodium usually glances off the female's belly, and the male rapidly backs up and thrusts again. Thus the analogue to the courting run is a series of from 4 to 20 thrusts at a more or less stationary female, executed at a rate of about one per second. The male often crosses to the other side every 2 or 3 thrusts. An occasional thrust seems to penetrate the vent, so that the fish cling together for 2 or 3 seconds. When they part from such a contact, they are likely to dart away as if startled. The male often dashes around the tank like a trapped housefly.

These lasting contacts are quite infrequent. One male made approximately 20 sequences, each averaging 10 thrusts, in as many minutes, without achieving a lasting contact. An examination of Table 1 will show that, although one or more series of thrusts were made in about half the sessions, lasting contacts occurred in only a quarter of them. Since there was rarely more than one lasting contact per session, compared to 50–300 thrusts, the inaccuracy of the gonopodium thrust is apparent.

But even the appearance of a lasting contact is no assurance that mating has been achieved. It is possible that the gonopodium may become caught in one of the ventral fins, preventing immediate separation. At any rate, out of 16 lasting contacts observed, only 1 yielded a batch of offspring. This contact differed in no apparent way from the other 15. It is possible that some of the unsuccessful contacts failed to impregnate the female and that others were vitiated by later abortions. But, at any rate, the act of mating seems to be as inaccurate as it is persistent.[4]

The male thrust usually comes as the culmination of a long courting sequence. In a typical session the fish are quiet for a few minutes after they are put in the tank. Gradually they start exploration, and after 5 or 10 minutes the male is likely to pose in front of the female, fins quivering and body bent in a sigmoid fashion. This is called the "male pose." It is interspersed with nipping (as in the Swordtail) and with periods of grazing on

[4] Cf. Clark, Aronson, and Gordon (1948). This abstract appeared after the present paper had been prepared for publication. These authors made a distinction between "jabs" and "long copulations," corresponding to our "thrusts" and "lasting contacts." Using a smear technique for the detection of sperm, they found that the jabs never resulted in insemination, whereas long copulations were successful 98 per cent of the time in Platies and 39 per cent in Swordtails.

the top or bottom. Over a period of 5 minutes the frequency and intensity of this behavior increases, until the male is in constant motion, alternating between posing, nipping, and "backward swim." The combination of a pose and a backward swim is clearly similar to the frequently noticed "arcing" of the Swordtail. Table 1 shows that its incidence in the Platy is actually higher than in the Swordtail.

TABLE 1

INCIDENCE OF MALE MATING RESPONSES

	Observation Series				
	P I*	P II	P III	Total	X†
No. of males	7	2	2	11	6
No. of females	7	5	4	16	8
No. of sessions	26	10	13	49	30
Nipping (per cent)	60	80	88	70	70
Male pose (per cent)	84	70	88	82	.....
Backward swim (per cent)	44	30	46	40	(18)
Jockey for position (per cent)	44	80	46	50	.....
Gonopodium flexion (per cent)	28	20	38	28	85
Gonopodium thrust (per cent)	56	55	42	50	55
Lasting contact (per cent)	22	30	33	25	03

* P I, P II, and P III represent three separate series of observations of mating in *Platypoecilus*.

† X presents comparable data on *Xiphophorus*, taken from Fig. 1. The percentages represent the proportion of all sessions in which each type of behavior was observed.

As courting speeds up, the posing and backward swimming occur behind the female as well as in front of her. The Platy may dash backward to the opposite end of the tank, flex his gonopodium, and then dash back for a nip or attempted thrust. During all this activity the female may remain relatively immobile; if so, the male is likely to initiate a series of thrusts, as described above. At the end of 5–20 thrusts, they break off, remain quiet for a half-minute, and then the male starts courting again. After five or ten series of thrusts, distributed over 10 minutes, the male gradually slows down, and they both remain quiet or feed on the bottom.

On the other hand, the female may react to the early stages of courting by resting on the bottom or by continuous swimming. One of the most frequent of the female activities is a peculiar sliding up and down the glass wall. She presses her mouth against the glass, with her body pointing slightly upward and seems to attempt to swim forward. Thus she slides up the glass to the surface and then reverses her slope and slides her mouth down the glass wall. This behavior may go on for 10 or 15 minutes, broken only by swimming to another wall now and then. The male responds to this behavior by "jockeying for position" for a thrust. He follows her up and down, with his head either above or below her flank and his fins quivering. If she stops for a moment, or especially if she swims across the tank, he is likely to attempt a thrust; the female usually avoids the thrust by a change of direction or by a flip of the tail. Males have been observed to jockey for position almost continuously for a half-hour, without getting in a single thrust.

Jockeying for position probably involves more than its name implies. Several males repeatedly struck the dorsal fin

of the female as they crossed over her back, so that the behavior approached what we have called a "nip."

In Table 1 the percentage of sessions in which each of the above types of behavior has been observed has been tabulated for the three series of observations with *P. maculatus* (P I, P II, P III), and comparable data for *X. hellerii* have been added (from Fig. 1) where possible. It will be seen that the incidence of nipping was roughly similar in the three series. The male pose was not tabulated for the Swordtail, but it has a high incidence in all Platy series. Backward swimming was twice as frequent in the Platy as was the corresponding arcing in the Swordtail. Jockeying for position did not occur frequently enough to warrant tabulation in the Swordtail, but it appeared in half the Platy sessions. Isolated gonopodium flexion was observed with relative infrequency in the Platy; the variation in incidence in the three series suggests that it actually occurred more often than was noted in the protocol. The Platy is much more active than the Swordtail during courtship, and the isolated gonopodium flexion may have escaped notice during the frenzied jockeying, posing, and backward swimming.

The incidence of thrusts is roughly the same in all sets of observations, occurring in about half the sessions for both species. But the apparently successful lasting contact was observed much more frequently in the Platy than in the Swordtail. The discrepancy may be due to the fact that the tanks did not permit a long, uninterrupted courting run, which would handicap the Swordtail but not the Platy.

IV. Intergeneric Mating

It is well known (Innes, 1942; Gordon, 1947) that Platies and Swordtails will interbreed, yielding fertile hybrids. This crossing does not seem to occur in their natural habitat, for Gordon (1947) reports his failure to find hybrids, even when both species inhabit the same stream. But it is relatively easy to obtain such hybrids by keeping one female Swordtail in a tank with a male Platy. The reverse method is reputedly less likely to be successful.

Several sessions were devoted to observing both types of interspecies mating. In each case the male had been isolated for several months before the test periods. The males of both species showed their characteristic courting behavior; the females did not seem to participate. Nevertheless, the males of both species occasionally reached the level of excitation needed for making gonopodium thrusts.

Thirteen hybrids were obtained by keeping male Platies with female Swordtails. The usual excess of females (Gordon, 1947) was obtained. One of the 2 males fertilized 2 females, yielding a second generation. Several other females were back-crossed to Platy males.

Fourteen observation sessions were devoted to 4 male and 3 female hybrids. The females were the first generation and were old (3 years). The males were second generation, about 1 year old. Of the 4 males, 3 showed nips and gonopodium flexions. Only 1 made a series of gonopodium thrusts, and that during only one session. The female remained in a corner of the tank during these thrusts; there was no run. On several occasions it was clear that the female was taking the lead in the courting. But, despite the apparent lack of sexual drive on the part of the males, it is obvious that their father, a first-generation hybrid, was not sterile! It was unfortunate that he died before it was possible to observe his behavior under controlled conditions. As it is, too little mating was obtained to do more

than hint that the hybrids resemble the Platy rather than the Swordtail.

Five sessions were devoted to 2 male and 1 female back-cross to a male Platy. The behavior was roughly similar to that of the pure Platy.

V. Homosexual Behavior

In spite of the marked sexual dimorphism in these fish, sex is not so stable as one might expect. There seems to be reliable evidence (Van Oordt, 1925) that a female Swordtail, after bearing several litters, may spontaneously turn into a male. Cohen (1946) has shown that female Platies, fed pregneninolene from 2 weeks after birth, will pursue and attempt to mate with normal females. Further, he indicates that normal males will attempt to mate with males that have been treated with estrogen, but not with untreated males. Grobstein (1940) has reported that adult female Platies will develop a malelike anal fin, under the effects of testosterone proprionate. Beach also mentions several other similar studies in his book (1948).

But treatment with hormones appropriate to the opposite sex is not necessary to atypical sex behavior. Noble (1938) reports that he and Borne found that isolated males attempt to mate with males but soon learn to discriminate. During the present investigations we have seen Swordtail and Platy females perform every one of the male responses, except gonopodium flexions and gonopodium thrusts—all directed toward males. Similarly, males will occasionally court and even execute gonopodium thrusts at another male.

One observation session was devoted to observing each of two "pairs" of male Platies. All 4 males were mature and had shown normal mating behavior in earlier tests with females. They had been isolated in the battery tank for approximately 6 weeks after the previous experiment.

On the day of the present observation, the first 2 males were removed from the isolation stalls and placed in a 2½-gallon observation tank. Inside of 15 minutes M-22 posed in front of M-21 and then tried a few gonopodium thrusts, in typical mating fashion, directed at the other male's vent. M-21 darted away. One minute later they had completely changed roles. These alternating attempts at copulation continued for 10 minutes, interspersed with posing. M-22 was the more active in posing and thrusting, while M-21 frequently nipped M-22. The nipping rapidly became mutual, until they were whirling in a circular "dogfight," in which it was impossible to determine the aggressor.

The other pair was placed in the tank with a glass separating them. Five minutes later the glass was removed. In 20 seconds M-25 had cornered M-24 and made 3 typical gonopodium thrusts. This was repeated at 4 and again at 5 minutes after the start of the session. M-24 was stationary and accepted the thrusts as a female would. M-25 continued to show typical male nipping, jockeying for position, and thrusting, for a total of 15 minutes. After a short quiet spell, M-24 did some jockeying for a thrust position and may have attempted a thrust. The behavior continued in a somewhat confusing fashion for the remainder of the period.

Male mammals will frequently attempt to mount other males (Beach, 1948). One might have anticipated more rigid stimulus control of mating behavior in a lower species, such as the fish. But the results of this and the previous section show that isolated (and presumably sexually deprived) male fish will attempt to copulate with females of another species or with males of their own species.

This may be considered as an example of the effect of strength of drive on the extent of stimulus generalization. With moderate drive, a male will mate only with a female of his own species and only if she shows the appropriate responses. But, as the drive is increased by isolation, the mating response will be elicited by a wider range of stimulus objects. Incidentally, this principle would account for the failure to find hybrids in natural situations, for the appropriate female is normally present.

VI. Summary

The early phase of courting behavior in the male Swordtail consists largely of two types, *nipping* and *arcing*, both probably serving to identify the male and to test the receptivity of the female. On her part, the female may show posing or nipping, which serve to stimulate a lethargic male. There may well be other less obvious responses, such as movements of caudal or ventral fins, that signal readiness on the part of the female. The male flexes his intromittent organ several times before actual copulation is attempted, which may serve to get the capsule of spermatozoa to the end of the intromittent organ. Eventually he makes a *thrust*, with the organ aimed at the female's genital papilla. If the female responds by swimming forward, the thrust is repeated, and they progress across the tank in a co-ordinated series of thrusts and advances, called a *courting run*. The whole mating sequence is thus made up of interstimulation and response; it is *not* a purely male activity, from which the female tries to escape.

Mating in the Platy is roughly similar. The male is generally more active, especially in his constant posing, backward swimming, and jockeying for position. Gonopodium thrusts occurred in about half the sessions for both species. The female Platy remains stationary during a series of thrusts, unlike the forward motion of the typical Swordtail courting run. Lasting contacts, in which the gonopod may have penetrated the female's vent, occurred in one-fourth of the Platy sessions and much less frequently in the Swordtail; even in the Platy, only about 1 thrust in 200 resulted in lasting contact, and only 1 out of 16 lasting contacts resulted in offspring. The mating behavior in both species is thus an extremely hit-or-miss affair and makes up for its inaccuracy by its constant repetition.

After protracted isolation, a male of either species shows wide stimulus generalization; for he will attempt to copulate with other males or with females of the other species.

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THE EFFECT OF PRIOR RESIDENCE UPON DOMINANCE IN THE FISH PLATYPOECILUS MACULATUS[1]

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ALTHOUGH hierarchies based upon the dominance-subordination relationship have been reported for several species of fishes (Noble and Borne, 1938, 1940; Noble, 1939*b*; Noble and Curtis, 1939; Braddock, 1945; Greenberg, 1946, 1947; and others), insufficient information is available concerning the effect of prior residence in a geographical area upon the establishment of dominance. Noble (1939*b*) has stated, without publishing his evidence, that familiarity with an area gives a resident a decided advantage over a newcomer in the fish *Xiphophorus hellerii*. Braddock (1945) has noted that a newcomer introduced into an established hierarchy of *Platypoecilus maculatus* is usually relegated to the omega position for a short period. Greenberg (1946, 1947) has reported essentially the same phenomenon for immature specimens of the green sunfish, *Lepomis cyanellus*. Although all these examples suggest a possible advantage derived from prior residence, only that cited by Noble indicates that such actually exists. In the others the fate of the newcomers may have had other causes.

Territoriality is known in several species of fishes (Reighard, 1920; Breder, 1936; Langlois, 1936, 1937; Noble, 1939*b*; Noble and Curtis, 1939; Raney, 1940; Greenberg, 1946, 1947; and others) but is usually treated in connection with reproductive behavior or with the behavior of the young of species, wherein it is also associated with the reproductive behavior of the adults; and it is believed to be a result, rather than a cause, of dominance. The possibility of a relationship between territoriality and prior residence has not been investigated in fishes, although its existence should be suspected,

[1] I owe much to Dr. Bernard Greenberg and Dr. Karl Stiles, both of whom have read this paper critically. Errors of fact or judgment are, of course, the full responsibility of the author.

since both depend, at least in part, upon recognition of, and reaction to, a limited geographical area.

The present study was undertaken in order to discover whether prior residence predisposes toward dominance in *P. maculatus*.

THE EXPERIMENTAL ANIMAL AND ITS TREATMENT

Here as in previous work (Braddock, 1945) *P. maculatus* was used as the experimental animal. The fish were purchased from a commercial breeder. This species is ovoviviparous and is not known to be territorial. The thirty aquaria[2] used were duplicates of small aquaria previously employed (Braddock, 1945). When not under observation, the fishes were kept in a large aquarium[3] in the room in which all the observations were made. This aquarium contained a number of rooted plants. Pure, unchlorinated well water was obtained from the Michigan State College system via taps in the animal house of the department of zoölogy, where all observations were made. Two large windows facing north, one large window facing east, and four reflector lamps suspended from the ceiling furnished the light. A fairly constant water temperature of approximately 29° C. was maintained.

Before the experiments described below were undertaken, 50 contact-pairs were set up and were maintained intact for 7 days. The members of each of these pairs were then separated and kept in isolation for periods of time varying from 6 to 36 hours. When reassembled after 6 hours, the former dominance-subordination relationships were resumed without preliminary fighting in 10 of the 12 pairs so tested. After 12 hours, only 5 of the 12 pairs tested for this period reacted in this manner. After 24 hours, all 13 of the pairs tested underwent preliminary fighting, and the same was true after 36 hours. This work was done to determine in a general way the duration of social conditioning by contact with one other individual.

Since preliminary experimentation had indicated that social conditioning by contact with one other individual is lost after an isolation period of less than 48 hours, the fishes were isolated for that length of time prior to each set of observations. During the periods of isolation, 160 of the 320 individuals observed were kept in bare aquaria. Hereafter these will be designated the "intruders." The remaining 160, hereafter called "residents," were confined in aquaria containing large amounts of duckweed and *Elodea*.

An individual transferred from a bare aquarium into one containing plants or vice versa behaves differently from one that is returned to an aquarium which it has been occupying. In the latter instance the fish settles to the bottom for a few seconds and then resumes its former activities. Nothing in its behavior would enable an observer to recognize that anything has happened to it. In contrast, an individual transferred from a bare aquarium to one containing plants, or vice versa, settles to the bottom for a few seconds and then spends the next few minutes swimming about. It enters all regions of the aquarium. Thereafter, it stops swimming actively, and one cannot recognize from its behavior that it has been moved. The differences in behavior under the two circumstances just described are taken as evidence that the fishes are capable of recognizing such changes in environment as were involved in these experiments. Furthermore, the fact that fishes which are netted and returned to their own aquaria do not exhibit unusual behavior is taken as evi-

[2] Size of small aquaria: 30.5 × 26.5 × 26.5 cm.

[3] Size of large aquarium: 5 × 2.5 × 2.5 feet.

dence that this experience has no important effect upon them. Further evidence was obtained by netting members of contact-pairs and returning them to their own aquaria. This was done five times each for 50 different contact-pairs, and in no instance was there a change in the dominance-subordination relationship between any of them. In accord with these observations, each intruder was carefully and rapidly netted at the conclusion of its isolation period and placed in an aquarium containing plants and occupied by a resident fish.

Only homosexual pairs were used in these experiments. The omission of male-female pairs was deliberate and was done to avoid the introduction of the sex factor, known to be one of the determinants for dominance in this species (Braddock, 1945). Eighty pairs of males and 80 pairs of females were assembled for the experiments.

In half the pairs observed the intruder was larger than the resident; in the others the resident was larger. No quantitative measurements were attempted, but size differences were kept to the minimum that could be observed with certainty with the naked eye. While useful evidence might result from accurate measurements, the handling of the fishes in the process might affect their subsequent behavior, since these fishes are very easily injured. In all, there were 40 female pairs with the intruder larger and 40 with the resident larger. The male pairs were assembled in the same proportions. This was done because the size factor, known to be important in determining dominance in *Platypoecilus* (Braddock, 1945), could not be eliminated. Hence it was employed deliberately in determining the existence of the effect of prior residence. If the larger fish became dominant with the same frequency whether it was the resident or the intruder, no effect of prior residence could be recognized. On the other hand, with the effects of social conditioning and sex largely eliminated, any result not clearly attributable to size should be the result of prior residence. Although other factors, unrecognized as yet, affect the dominance potential in this species (Braddock, 1945), under the conditions of these experiments such factors should tend to neutralize one another.

The initial reactions of all pairs were observed carefully and recorded in writing. Only one pair was observed at a time. Data were assembled that indicated in each case: the first challenger; counterchallenges in response to the initial challenges; the first individual to retreat or be driven away; the first established victor; and the times at which these events occurred. Observations were also made on the 3 days immediately following the initial day, in order to record reversals in dominance relationships and other points of interest.

EFFECTS OF PRIOR RESIDENCE

In the experiments reported here behavior of a characteristic type occurred when the intruders were introduced. This was essentially the same, regardless of sex. Prior to the first contact reactions the intruders were more active than the residents. Each intruder tended to swim about actively until the resident was encountered. Conversely, the residents were more likely to remain in one place, usually at the top or in the corners of the aquaria. This lack of activity among the residents was especially noticeable in the males, a condition to be expected on the basis of their known tendency toward this type of behavior (Braddock, 1945).

Most of the initial challenges occurred within 5 minutes after the introduction of the intruders (Table 1). In order to be certain that the alert posture would not

be mistaken for challenging, which it somewhat resembles, a fish was not considered a challenger unless it approached the other individual and exhibited the full challenge pattern. A number of cases were recorded that were uncertain when this criterion was applied to them, since both individuals approached and challenged simultaneously, or so nearly so, that it was impossible to note which had taken the initiative. In Table 1 these are recorded under the heading "mutual." The material presented in Table 1 indicates that among the females the residents challenged first approximately three times as often as the intruders did. These proportions held whether the intruders were larger or smaller than the residents. Among the males the effect was even more pronounced. The resident males, when larger than the intruders, gave the initial challenges approximately eight times as often as did the latter; when smaller, four times as often. At this stage size had a greater effect upon the males. This is in accord with previous work (Braddock, 1945), where it was shown that the size factor is more effective in determining dominance among males.

TABLE 1

THE FIRST CHALLENGES

First Challenger	Resident Larger			Intruder Larger		
	Resident	Intruder	Mutual	Resident	Intruder	Mutual
No. cases ♀♀	28	9	3	25	8	7
Per cent ♀♀	70.0	22.5	7.5	62.5	20.0	17.5
No. cases ♂♂	34	4	2	29	7	4
Per cent ♂♂	85.0	10.0	5.0	72.5	17.5	10.0

After the first challenges had been given, the fishes that were challenged responded in one of two ways. They submitted at once or met the initial challenges with counterchallenges. The latter response was the characteristic one (Table 2). Cases in which the first challenge appeared to be given mutually are omitted from the table, since it was impossible to judge whether they represented instantaneous counterchallenges on the part of one of the participants or true mutual reactions. While both residents and intruders met the initial challenge with a response in kind more often than not, the residents more frequently reacted thus. This was consistent regardless of size or sex. Thus the residents were more likely not only to initiate aggressive behavior but also to respond aggressively when the intruders did so. Because the intruders infrequently challenged first, it was impossible to make an adequate comparison of the counterchallenges between males and females.

Observations were also made to learn in how many instances one of the members of a contact-pair actually retreated or was driven away after the first period of challenging and to note whether the residents or intruders were more likely to be forced into retreat (Table 3). In a few cases this occurred without counterchallenging; in more, after a short or prolonged period of counterchallenging; and in a few after a small number of nips was given or exchanged. Among the females the intruders retreated three times as

frequently as did the residents when the latter were larger; twice as often, when the residents were smaller. The male intruders also retreated more often than the residents: approximately four times as often when the residents were larger and three times as often when they were smaller. There were a number of instances in which neither fish retreated. These occurred when both individuals were extremely belligerent, and both backed off only to resume fighting after a period of inactivity.

Retreat after the initial period of aggressiveness did not always result in permanent defeat of the individual that gave way. Whatever might occur under natural conditions, the fishes were forced to meet repeatedly because of the small size of the aquaria used. At such meetings nipping occurred, and this continued until a stable relationship was established (Table 4). A stable relationship was judged to exist when one individual submitted to the nips of the other over a period of several meetings without counterchallenging or nipping in return. The effect of prior residence had decreased in relative importance at this stage. When the intruders were larger than the residents, they won approximately 50 per cent of the victories; when smaller, only 15–17 per cent. Since the size of the fishes did not change during the experiment, it must be assumed that even a short acquaintanceship with an area gives an intruder an advantage that is lacking when it is first introduced. It should not be supposed, however, that the effect of prior residence had disappeared altogether at this stage. If such had been the case, the larger individuals would have

TABLE 2

COUNTERCHALLENGES IN RESPONSE TO THE INITIAL CHALLENGES

Counterchallenges	Resident Larger		Intruder Larger	
	Resident	Intruder	Resident	Intruder
No. first challenges received ♀♀	9	28	8	25
No. counterchallenges ♀♀	8	18	6	15
Per cent of first challenges met with counterchallenges ♀♀	88.8	64.3	75.0	60.0
No. first challenges received ♂♂	4	34	7	29
No. counterchallenges ♂♂	3	21	7	21
Per cent of first challenges met with counterchallenges ♂♂	75.0	61.8	100.0	72.4

TABLE 3

RETREATS AFTER PERIOD OF INITIAL CHALLENGING

Retreats	Resident Larger			Intruder Larger		
	Resident	Intruder	Neither	Resident	Intruder	Neither
No. cases ♀♀	8	27	5	11	22	7
Per cent ♀♀	20.0	67.5	12.5	27.5	55.0	17.5
No. cases ♂♂	8	30	2	9	26	5
Per cent ♂♂	20.0	75.0	5.0	22.5	65.0	12.5

won with the same frequency whether they were residents or intruders. The female residents, when they were larger, won 85 per cent of the victories and the larger female intruders only 50 per cent. The males followed the same pattern.

After the first stable contact-pair relationships were established, conditions tended to remain unchanged during the remainder of the 4 days of observation. This was in agreement with previous findings (Braddock, 1945), where the mean duration of such relationships between two individuals was found to be 9.9 days for females and 5.8 days for males when the fishes were assembled in groups of four. Reversals were relatively few, and those that occurred commonly did so within the first 24 hours after the experiments were started. Only 4 of the 26 reversals occurred later. Nineteen of the 26 cases involved the eventual victory of the larger individual; only 7 favored the smaller. This may indicate that these so-called "reversals" were really delayed first victories. The cause of the delay might have been the nearly equal belligerence of both members of each contact-pair. However, reversals from smaller to larger were more frequent where the intruder was the larger fish. The larger residents were favored from the outset and, in most cases, established their superiority early.

During the periods of observation, no tendency was noted for any of the individuals to favor or shun any parts of their aquaria, to avoid the other fish in any parts, or to be more dominant or submissive in any parts.

TABLE 4

THE FIRST STABLE RELATIONSHIPS

Victor	Resident Larger		Intruder Larger	
	Resident	Intruder	Resident	Intruder
No. cases ♀♀	34	6	19	21
Per cent ♀♀	85.0	15.0	47.5	52.5
No. cases ♂♂	33	7	20	20
Per cent ♂♂	82.5	17.5	50.0	50.0

DISCUSSION

The possible effect of prior residence was tested against a known effect of size, and it has been shown that the former exists and confers an added advantage upon the resident fish. This is true for both sexes. The resident individual is more likely to give the first challenge, more likely to respond to an initial challenge with a counterchallenge, less likely to retreat first, and is the probable first established victor. Also there is some reason to believe that the prior-residence effect can delay the eventual victory of an otherwise superior individual. There are indications that the determining effect of prior residence gradually diminishes with time and that other factors may predominate as the intruder becomes more familiar with the area. This is true in regard to size, since the larger individuals are more frequently dominant as time passes. Also factors other than prior residence and size are operative, since a few cases occurred in which the smaller individuals were more aggressive even when they were the intruders.

Under natural conditions a group of

individuals might be confined in a limited but uncrowded area. This could occur during a dry season, when the streams might become a series of pools. Under such conditions the prior-residence effect could operate, and the crisis would probably occur at the time of the first retreat. At this stage, even under the artificial conditions of these experiments, the resident individuals exhibited a definite superiority. In the normal course of events an intruder might be driven away at this point. If it should return immediately, it would probably be driven away a second time; after a lapse of time the initial reactions would probably be repeated from the beginning. This point should be clarified, since it raises a significant set of alternatives. Briefly stated, the problem involves the relative importance of hierarchical behavior and the prior-residence effect under natural conditions. The prior-residence effect would seem to inhibit the formation of hierarchies, since the intruders would be driven away before nip-right relationships could be formed. Perhaps this might lead to a nip-dominance situation wherein the residents would be dominant within their places of residence but not elsewhere. Such an interpretation assumes that the prior-residence effect is an aspect of territoriality as that term is generally understood. If this interpretation is correct, true hierarchies should occur only under very crowded conditions.

The other alternative would recognize hierarchical behavior as the norm. If this is true, the prior-residence effect may occur only under unusual and possibly unfavorable conditions, when it might prevent overcrowding by keeping the individuals thinly spread out over the maximum amount of available space, thus making it more probable that they would find food and avoid their enemies. Such evidence as is available favors this viewpoint; for *Platypoecilus* in large aquaria do form hierarchies. Conditions are uniform and favorable throughout, and individuals rove the entire available space and have not been observed to maintain residence or territories in any part thereof. No examples of nip-dominance behavior have been observed even in large aquaria.

The relationship between the prior-residence effect and territoriality is partially a matter of definition. Noble (1939*a*) defines a territory as "any defended area." Greenberg (1947) restricts this to the defense of a subarea within a larger area, the subarea having more or less definite boundaries. On the basis of Noble's definition as amended by Greenberg, there is some justification for an attempt to include the prior-residence effect under territoriality. Certainly, the area represented by the entire small aquarium is defended. This represents a departure from Greenberg's definition. He would exclude an area with limits artificially set, i.e., otherwise than by competing individuals. However, there seems no reason to believe that limits are established only by the individuals themselves under natural conditions. A subarea could consist of that part of a larger area where there was available more food or better protection. The fact that Greenberg's sunfish tended to defend the corners of the aquaria, where the defensive situation is more favorable, is in support of this supposition. Furthermore, there must be limits to the extent of the area over which the prior-residence effect can operate. In many birds (Odum, 1941; Collias, 1944) prior residence is believed to confer an advantage in acquiring and holding territory. While no such phenomena have as yet been reported for fishes, future work may uncover facts of this nature. It should be noted, however, that neither Odum nor Collias states

that prior residence is identical with territoriality.

It is noteworthy, however, that the prior-residence effect differs in several important respects from known examples of territoriality in fishes. In the first place, true territoriality strengthens the position of the individual holding an area as time passes, while the effect of prior residence, as here demonstrated for *Platypoecilus*, becomes less with the passage of time. This may be due either to a loss of advantage to the resident fish or to an increase in dominance potential in the intruder. Second, unlike true territoriality, prior residence is a cause rather than an effect of dominance. If the situation in the green sunfish (Greenberg, 1947), in which "distinct levels of aggressiveness" appear to inhibit the formation of territories, also applies to *Platypoecilus*, the prior-residence effect would seem to militate against territoriality, since it reinforces the aggressiveness of the resident and thus tends to increase the difference between it and the intruder in this respect. Thus under crowded conditions the prior-residence effect would favor the eventual formation of hierarchies but not of territories. Related evidence includes the fact that territoriality has not been reported for any ovoviviparous species of fish. Also true territoriality is usually associated with nip-dominance, rather than nip-right, behavior. At present, the weight of evidence favors the view that the prior-residence effect, while having a few features in common with territoriality as the latter is generally understood, presents relatively more differences.

More information is required concerning the causes underlying the effect of prior residence. While the belligerence of various intruders differs, their overt behavior indicates that they react directly to a strange environment, since they behave in much the same manner whether another fish is present or not. The causes underlying the behavior of the residents are less clear. They react to the intruders and are usually belligerent, even when the intruders are extremely truculent. Since the residents react in the same manner, regardless of the relative belligerence of the intruders, one might well suppose that the familiar environment is a factor in their reactions. It may well be that because they are free from the necessity for adjustment to a new environment they can devote their entire attention to the intruders.

SUMMARY

1. In *P. maculatus*, prior residence in an area confers upon an individual a greater potential for dominance than it would otherwise have.

2. The effect of prior residence applies to both sexes.

3. The effect of prior residence operates most effectively during the initial contacts between individuals and becomes less important as time passes.

4. The effect of prior residence is in competition with the size factor when the intruder is larger than the resident, and the size factor may prevail.

5. The relationship, if any, between prior residence and territoriality is not clear.

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DISGORGING OF FOOD TO THE PUPPIES BY THE LACTATING DOG

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IN THE course of an experiment dealing with the hormonal regulation of micturition behavior in dogs, we observed that three females, during the final stages of lactation, regularly disgorged food before their young. A description of these observations may be of value in extending our knowledge of the maternal behavior in the dog.

MATERIAL AND METHODS

Three multiparous dogs were used: Dog F was a fox terrier, weighing 6 kg., with a litter of 5 puppies; Dog S, a mongrel of 6 kg., with a litter of 5 puppies; and Dog M, also a mongrel, of 9 kg., with a litter of 4 puppies.

The females were fed milk in the morning and at 1:00 to 2:00 P.M. a meal of meat, which was sometimes mixed with corn meal. The food was always placed at a distance of 5–7 meters from their nests and litters.

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TIME RELATIONSHIP BETWEEN THE DISGORGING BEHAVIOR AND LACTATION

Table 1 shows the chronological relations between several elements in the pattern of maternal behavior. Initial decline in lactation was associated with several changes in overt responses. The females spent a shorter time in the nest and less frequently adopted the bodily posture which facilitates suckling. In addition, of course, actual suckling decreased. It is apparent that disgorging re-

TABLE 1

CHRONOLOGICAL RELATION BETWEEN VARIOUS ELEMENTS IN THE MATERNAL BEHAVIOR PATTERN

Dog	Disgorging First Observed	Disgorging Last Observed	Lactation Begins To Decline	Weaning Complete
F.....	24*	39	23	36
M.....	21	56	24	46
S.....	22	34†	.....	.....

* All figures represent number of days postpartum.
† Observation was discontinued before the response disappeared.

actions are initiated about the time that lactation starts to decline and continue a few days after suckling has ceased.

DESCRIPTION OF THE BEHAVIOR

The behavior was observed daily, and several moving pictures were taken at this department. Shortly after ingesting a meal the females returned to the homeroom, usually going directly to the nest and there vomiting up approximately one-third to one-half of the food they had recently consumed. Disgorging was accompanied by energetic contractions of the diaphragm and abdominal muscles. Generally, this activity occurred within 15 minutes after eating, the delay varying from 2 to 36 minutes. A few times the females were observed to vomit twice, but as a rule the response occurred only once after each feeding. Sometimes a bitch ate all her food and then disgorged part of it. In other cases part of the meal was eaten, and, after vomiting near the puppies, the mother returned to finish eating.

Before vomiting, Dog M scratched at the nest and then displayed rhythmic head movements like those of a dog burying a bone; but, if her young had been suckling just before the female disgorged, these preparatory acts were omitted.

In all cases the puppies promptly and voraciously devoured the disgorged material. Females F and S never ate any of their own vomit, but Dog M ate almost all the vomited mass on 3 of 31 observed instances.

The female's behavior after feeding was definitely affected by the presence of the puppies. On five occasions the litter of Female M was removed from the homeroom for 23–25 hours. While separated from her puppies the bitch was restless and paid little attention to the nest. She was closely observed for 30–45 minutes after each feeding, and disgorging never occurred. The behavioral preliminaries to this act were likewise lacking. When she was led to the puppies, which were in another room, the female vomited within 2–5 minutes.

Attempts to elicit vomiting in the absence of the young were unsuccessful, even though various substitutes were offered. Young kittens and toy dogs similar in size to her own litter were employed as stimulus objects. The female sniffed the dog models and lifted two of the four kittens out of the nest, holding them gently between her jaws in the same manner as she treated her own young. However, disgorging occurred only when she was led to her own puppies. Apparently, some stimulus or stimuli associated with the puppies are essential to the occurrence of postfeeding vomiting.

These observations emphasize the significance of the disgorging as a response to an environmental stimulus. Furthermore, all three dogs were healthy, and no vomit had been detected in conditions other than those described in this paper. There is some indication that the act of suckling immediately preceding disgorging may have a facilitating effect, although it is not essential to the behavior under discussion. As noted above, when suckling occurred, the ordinary preliminaries to disgorging were omitted.

Another method of feeding the young was observed. On the same day that disgorging behavior commenced, Dog F began to transport meaty bones to the nest and to leave them with the puppies, at the same time retiring to another spot with bones which formed her own meal. This type of response was exhibited from the 24th to the 38th day postpartum.

The behavior of Female M was more complex. This female began to carry bones to the nest 38 days after parturi-

tion, ate in the nest, and did not allow her offspring to share the food, although after feeding she often disgorged part of the meal and permitted the puppies to eat it. Unwillingness to let the young chew on the bones gradually decreased, and by the 49th day postpartum the puppies were allowed to share their

FIG. 1 FIG. 2

FIG. 1.—Dog M eats the meal
FIG. 2.—Ten minutes after, M runs to the nest and begins scratching

FIG. 3 FIG. 4

FIG. 3.—Two minutes later, disgorging
FIG. 4.—The young eat the disgorged material. All pictures taken on the 27th day postpartum

mother's food. Ten days later, bones were transported to the nest and left with the young while the mother went to another place to eat her own food.

DISCUSSION

Any interpretation of the behavior described here must take into consideration both nervous and hormonal factors. The established relationships between maternal performance and certain hormones, considered in conjunction with the simultaneity of such behavior with lactation, suggest a common endocrine basis. A remote analogy with crop-sac secretion and parental reactions in the pigeon is obvious, but direct experiments with prolactin and other hormones are needed before conclusions can be drawn.

The literature on dog behavior contains, so far as we can ascertain, no reference to the disgorging behavior, although this behavior is known to practical breeders. This may signify that this behavior has been overlooked or that the usual domestic conditions are not always favorable to its manifestation.

The disgorging behavior of the canine female during lactation is not peculiar to dogs, for observers have recorded its oc-

currence in the wolf. Indeed, in the natural or wild condition this behavior might well be indispensable. To eat, carnivores must hunt, and a recently weaned puppy is obviously unable to trail, chase, and conquer prey, which are activities necessitating an advanced state of neuromuscular development as well as, in all probability, a considerable amount of learning.

In addition, the mother's milk available during the last stages of lactation does not provide sufficient nourishment for the young. This is confirmed by the fact that dog breeders find supplementary feeding necessary during the final weeks of the suckling period.

It would be of interest to discover whether similar behavior can be found in carnivores other than the Canidae.

SUMMARY

A description is given of the behavior of three lactating dogs which, around 3 weeks postpartum, began to disgorge food for the puppies. The vomited material was voraciously eaten by the young. This behavior was regularly maintained up to 3–10 days after weaning, disgorging occurring in most cases within 15 minutes after the meal.

Disgorging did not occur if the puppies were temporarily separated from the mother. Another method of feeding the young was observed: the mother transported meaty bones to the nest and allowed the puppies to chew them. A brief discussion is made, suggesting the possibility of an endocrine control of this behavior and emphasizing its importance in wild life.

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AQUATIC RESPIRATION IN THE MUSK TURTLE

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As is well known, a number of aquatic species of turtles can survive relatively long periods of time when completely submerged in water. Some, in fact, voluntarily remain submerged for such periods. During submergence they often show more or less rhythmical movements of the throat, reminding one of the breathing movements of fishes and various Amphibia. It can be demonstrated by the use of carmine particles that these movements cause water to move in and out of the buccopharyngeal cavity. It has long been suspected that such movements provide a respiratory current of water or, if the turtle is on land, of air and that a certain fraction of the total gaseous exchange of the animals might occur through the mucous membrane of the mouth and throat. It has also been thought that the throat movements are an essential part of the mechanism of lung respiration, comparable to the case of the frog, in which air is forced into the lungs by elevation of the hyoid apparatus. The latter function of the throat movements was ruled out by Mitchell and Morehouse (1863) many years ago and has recently been discredited anew by the observations of Hansen (1941) and those of McCutcheon (1943). There is some evi-

dence to support the view that the throat movements provide aquatic turtles with a respiratory current of water when they are submerged. Those familiar with the older literature on turtle respiration will recall the observations of Gage and Gage (1886) on the soft-shelled turtles, *Amyda mutica* and *Aspidonectes sperifer*. These turtles voluntarily spend long periods of time under water, and, while submerged, show rhythmical throat movements occurring at a rate of approximately 16 per minute. The mucous membrance of the throat is beset with villi-like projections, highly vascularized. Gage and Gage showed experimentally that a significant gas exchange occurred in submerged animals, and they attributed all but a small fraction of it (which took place through the skin) to buccopharyngeal respiration. It was their belief that such a respiratory mechanism contributed significantly to the ability of these animals to remain under water.

The musk turtle, *Sternotherus odoratus* (Latreille), is another example of an aquatic turtle that voluntarily spends fairly long periods of time under water, and, if forced to stay submerged, can do so, without loss of life, for hours. While submerged, it, too, shows the characteristic movements of the throat described above for the soft-shelled species. The movements, however, are quite variable in rate and periodicity. One gets the impression that they are more vigorous and rapid at a time when the animal is moving about and tend to occur less often and in a less predictable way when the animal is perfectly quiet. Unlike the soft-shelled turtle, there are no discernible villi-like projections in the throat, and there exists no more than the usual vascularization of the mucous membrane.

As far as the author is aware, no one has ascertained whether or not the ability of the musk turtle to remain submerged is dependent upon buccopharyngeal respiration, as seems to be the case for the soft-shelled turtles described by Gage and Gage. The opportunity for making such a study presented itself while the author was a research associate at the American Museum of Natural History. The Laboratory of Experimental Biology, then under the directorship of the late G. K. Noble, had available a large supply of musk turtles. It will be the purpose of this paper to report the outcome of a series of observations made upon these animals.

It is the wish of the author to acknowledge his indebtedness to the late G. K. Noble and to the American Museum of Natural History for making available the facilities of a laboratory. He also wishes to thank Mr. Moses Wurm for extensive technical assistance.

METHODS AND RESULTS

A. *Total aquatic O_2-consumption.*—In all work to be reported, O_2-consumption only was used as a measurement of respiratory exchange. To determine how much O_2 was consumed by submerged musk turtles, animals which had been marked and fasted for a week were sealed away from air in large cylindrical museum jars containing a known volume of respirable water with a known O_2-content. These jars were then placed in a thermostatically controlled water bath at a temperature of 25° C. for a fixed period of time (usually about 4 hours). At the termination of the fixed time period the water in the jars was reanalyzed for dissolved O_2 and the animal's weight and volume determined. From the data on initial and final O_2, the respirable volume of the water, the weight of the animal, and the experimental time, the O_2-consumption per kilogram per hour can be

calculated for each animal by using the following simple equation:

$$\frac{1{,}000\,RO}{GT} = \text{O}_2\text{-consumption in ml/kg/hr},$$

where R is the respirable volume in liters, O is the O_2-consumption (difference between initial and final O_2) in milliliters per liter, G is the weight of the animal in grams, and T the experimental time in hours. Oxygen determinations were made according to the Birge-Juday modification of the Winkler technique. Initially, the Stewart permanganate modification was used, but later experience taught that the difference between the two methods was insignificant, and the Stewart modification was abandoned for the less tedious Birge-Juday technique.

In all, 18 different animals were used for this part of the work. In a number of instances several determinations of the aquatic O_2-consumption were made repeatedly on the same experimental animal over a period of 6–8 months. The results for all determinations are recorded in the second column of Table 1. The mean aquatic O_2-consumption based on 25 determinations, without regard to animal, is 9.50 ml/kg/hr. The range of single measurements extends from 5.06 to 13.88 ml/kg/hr, and their standard deviation from the mean is ± 1.99 ml/kg/hr.

B. *O_2-consumption with head masked.*—In Part A, where the animals were simply submerged, it could not be decided whether or not the O_2 consumed from the water was taken out solely via buccopharyngeal respiration, for some may have been absorbed by the skin of the animal. In order to get information on this point, a fairly thick rubber mask was placed securely over the head of the animals to prevent buccopharyngeal ventilation by currents of water. The animals were then subjected to the same procedure as that outlined in Part A. Twenty-one single determinations were made with head masked on 14 different animals. Any O_2-consumption observed was presumed to be due to the absorption of O_2 by the skin of the animals. The results are presented in the third column of Table 1. The O_2-consumption for single determinations, without respect to animal, is 5.50 ml/kg/hr. The range of single determinations extends from 3.03 to 7.92 ml/kg/hr, and their standard deviation from the mean is ± 1.62 ml/kg/hr.

C. *Aquatic O_2-consumption with body masked.*—To get further confirmatory information on the role played by buccopharyngeal respiration in the total aquatic O_2-consumption, the reverse procedure of Part B was utilized. A tight-fitting rubber mask covered with paraffin was placed completely over the animal, leaving only the head and part of the neck free. The animals were again subjected to the procedure of Part A. In this case any O_2 consumed from the water was presumed to be due to buccopharyngeal respiration (throat movements were not interfered with by the body mask); for the small amount of skin still exposed on the bony head and part of the neck could be considered a negligible source of O_2-absorption. In all, 11 determinations were made on 10 different animals. The determinations are shown in the fourth column of Table 1. The mean O_2-consumption with this technique is 2.75 ml/kg/hr. The individual measurements range from 1.40 to 5.82 ml/kg/hr and have a standard deviation from the mean of ± 1.53 ml/kg/hr.

D. *Total air O_2-consumption.*—To make a fair decision as to whether the O_2 consumed by the animal when submerged is of any significance in enabling it to withstand submergence, it would be necessary to know approximately how

much O_2 is consumed when the animal's respiratory devices are working at normal capacity, as they might when lung respiration is not impeded. Accordingly, certain of the animals used in the previous experiments were placed individually in an air respirometer patterned after the model described by Krogh (1914), and the O_2-consumption was measured over a period of time approximately equivalent to that allowed for the submergence experiments (4 hours). To enable direct comparison, the results obtained were again expressed in milliliters of O_2 con-

TABLE 1

RESPIRATION IN THE MUSK TURTLE

(Temperature 25° C.)

Animal No.	Total Aquatic Respiration (Cc/Kg/Hr)	Respiration with Head Mask (Cc/Kg/Hr)	Respiration with Body Mask (Cc/Kg/Hr)	Air Respiration (Cc/Kg/Hr)
1	9.20	7.82	1.93	93.4
	8.35	5.26	2.69	92.4
	.....	.....	.....	141.0*
	.....	.....	.....	130.2*
2	11.20	6.16	5.24	66.4
	9.24	.....	.....	.....
3	7.97	3.22	.....	61.0
	9.32	4.14	.....	.....
	.....	3.03	.....	.....
4	9.70	7.92	.....	20.9†
	11.40	3.50	.....	.....
5	11.12	6.06	1.40	.....
6	11.02	.....	.....	.....
7	11.00	7.73	.....	.....
	9.52	6.38	.....	.....
9	10.41	7.71	.....	.....
	11.22	7.02	.....	.....
10	12.83	6.05	1.50	66.0
12	9.03	4.93	1.49	56.9
	7.50	.....	.....	91.8
	.....	.....	.....	95.2
	.....	.....	.....	79.7
13	8.21	4.15	.....	38.4
	.....	.....	.....	21.2
	.....	.....	.....	56.2
14	6.78	.....	3.68	97.2
15	8.55	5.51	.....	51.0
16	5.06	3.67	2.54	60.1
17	13.88	6.95	5.82	71.9
	.....	.....	.....	62.9
18	9.66	5.46	2.11	50.6
19	6.51	.....	.....	.....
20	8.82	.....	1.87	.....
Mean of single measurements	9.50	5.50	2.75	71.1
Standard deviation of single measurements	1.99	1.62	1.53	31.23

* Very active during respiratory observations.

† Extremely quiet all during period of observation.

sumed per kilogram per hour. By the time that this stage of the experiments had been reached, not all the original animals were alive. Thus measurements could be made upon only 12 of them. Twenty-one single measurements were made on these 12 animals. The results are shown in the last column of Table 1. The mean air O_2-consumption based on the single determinations is 71.1 ml/kg/hr. The range of the individual measurements extends from 20.9 to 141.0 ml/kg/hr, and their standard deviation is ± 31.2 ml/kg/hr. This is somewhat large, but to those familiar with respiratory measurements made on animals whose activity is highly variable during their sojourn in the respirometer, the magnitude is not too surprising. In all these experiments, no matter whether the animals were in air or in water, no attempt was made to control their activity. Some would remain quietly in the respirometer for long periods, but others would engage in frantic efforts to escape, with relatively short lapses into quietude.

In order to strengthen the mean of the air measurements, 14 additional determinations were made on 8 turtles that had not been used in the previous experiments but were, in other respects, treated in the same way as were the original 12 referred to above. The mean of the measurements for these turned out to be 85.3 ml/kg/hr, with individual values ranging from 43.1 to 118.9 ml/kg/hr. Bringing these measurements in with the former changed the mean to 77.1 ml/kg/hr. The standard deviation for the 35 measurements is ± 28.2 ml/kg/hr.

DISCUSSION

The results of this investigation indicate that the aquatic respiration of these turtles will, on the average, provide for only about one-eighth of the total O_2 consumed by them when they are in air. They show, further, that the buccopharyngeal moiety provides for only about one-twenty-fifth of the total air O_2-consumption. On the basis of these figures it seems unlikely that aquatic respiration, especially the buccopharyngeal fraction, explains the ability of these animals to withstand submergence as long as they apparently can. Other factors must be involved, however. It would be interesting to compare the O_2-"stores" of aquatic turtles with the terrestrial varieties. It may be that they are larger. Irving (1939) made an attempt to compare diving mammals with nondivers in the hope that striking differences in O_2-stores might be shown. His findings were disappointing, for any difference that existed seemed too slight to account for the ability of the divers to remain submerged. Yet, in the turtle, conditions may be different, and it might be profitable to investigate such factors as (*a*) the ratio of lung volume to body-weight; (*b*) the total blood volume; (*c*) the blood O_2-capacity; (*d*) the efficiency of the buffering mechanisms; and (*e*) the ability of the nervous and humoral mechanisms to shift blood flow to vital organs, such as the brain, and away from less vital regions during a period of submergence. As far as the author is aware, sufficient data of this sort are not available at the present time to enable such a comparison.

In the course of these investigations the author never ceased to marvel at the ability of these animals to withstand submergence. The normal 4-hour period of submergence in the aquatic respiratory experiments seemed to have little lasting effect on the viability of the animals. They usually became less active near the end of the period. Upon removal from the

respiratory chambers they obviously were weakened somewhat and were less reactive to stimuli; but after a short period of hyperpnea they recovered promptly. On two occasions, owing to the author's inability to remove the covers from the museum jars, two turtles were forced to remain submerged in the chambers overnight. When they were finally released, analysis of the water showed that the dissolved O_2 had gone down to about 0.2 ml/liter. Neither animal survived, but reflex movements of the legs of one animal could still be elicited after 27 hours of submergence.

As to the function of the underwater throat movements, the author is forced to believe that they subserve only an insignificant and fortuitous respiratory role. Opinion is growing that such movements in any of the turtles are primarily concerned with olfaction (Vos, 1936; McCutcheon, 1943). In the soft-shelled turtles studied by Gage and Gage (1886) in which buccopharyngeal respiration seems to be significant, it is quite probable that this represents a secondary adaption. If the throat movements of turtles were normally serving a respiratory function, one might expect their rate and amplitude to be increased in the early stages of respiratory stress. As far as the musk turtle is concerned, there is no evidence to indicate this. The throat movements were most prominent when the turtle moved about in the respiratory chamber. If the animals were quiet, they would appear at unpredictable times, and there would be long periods when none was discernible. On the basis of the more common association of throat movements with the general exploratory activity of the animal, the author is inclined to the opinion that they subserve primarily a chemical or olfactory function and that any respiratory function is fortuitous or secondary. One could infer from the observations on the musk turtle that they represent a kind of "sniffing" mechanism, brought into play as the turtle explores its surroundings.

SUMMARY

1. O_2-consumption, as it occurs in the musk turtle when completely submerged in water, has been compared with the normal O_2-consumption of the animal in air. The purpose of the comparison was to evaluate the role that buccopharyngeal respiration might play in helping the animal to remain submerged.

2. It was found that the total aquatic O_2-consumption, on the average, was equivalent to about one-eighth of the O_2-consumption of the animal in air.

3. The buccopharyngeal fraction of the total aquatic O_2-consumption was found to be equivalent to only about one twenty-fifth of the air O_2-consumption. The remainder of the O_2 consumed under water occurs by absorption through the skin.

4. It is concluded that aquatic respiration, particularly the buccopharyngeal fraction, contributes insignificantly to the ability of these animals to remain submerged and that other factors must be sought.

5. It is suggested that the throat movements under water, which lead one to suspect buccopharyngeal respiration, are primarily concerned with olfaction, as has been suggested by other authors. The small respiratory function that they subserve is probably quite fortuitous in the musk turtle but may represent a secondary adaptation in some of the other aquatic species in which the function seems to be more significant.

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COMPARISON OF THE ANTITRYPTIC ACTION OF THE LARGE ROUNDWORM OF THE CHICKEN (ASCARIDIA GALLI) AND THE COMMON EARTHWORM (LUMBRICUS TERRESTRIS)[1]

L. D. BUSHNELL AND L. E. ERWIN

DURING a recent study of the antitryptic action of *Ascaridia galli* by Bushnell and Erwin (1948), interest was aroused in determining whether organisms which were not compelled to live in the presence of tryptic enzymes would possess an antitryptic property comparable to that developed by organisms which were thus exposed. For this reason the common earthworm (*Lumbricus terrestris*) was selected for comparison with *A. galli*.

The antienzymatic property is commonly measured by the ability of a serum or tissue extract to inhibit the action of trypsin and for this reason is spoken of as the "antitryptic action."

REVIEW OF LITERATURE

Many studies have been made on the antitryptic action of healthy and diseased tissues, and numerous theories have been advanced to explain its nature. Hildebrandt (1893) was the first to describe the antitryptic action of blood serum and its power to inhibit the proteolytic actions of leucoproteases. This observation was confirmed by Fermi and Parnossi (1895) and was described in detail by Opie (1905). The nature of these inhibiting compounds is unknown, and numerous theories have been advanced to explain their action.

Most investigators state that these antienzymes are not specific. The antitryptic action of serum has been attributed to a lipoid-protein substance; to certain colloids which modify surface tension; to the particular configuration of the protein molecule; and to the presence of lipoids and soaps by Jobling and his co-workers (1914).

Burge and Burge (1915) suggested that the protection of parasites and perhaps the mucosa of the alimentary tract itself from digestion by proteolytic enzymes depends upon the active oxidative properties of the tissues in destroying the enzymes. Bayliss (1924) stated that in his experiments the antienzyme action was mainly due to the acidity of the solu-

[1] Contribution No. 240 of the Department of Bacteriology, Kansas Agricultural Experiment Station.

tion, and he pointed out that such a condition might be important in inflammation. It is also reported that the intestine is protected less by an antienzyme than by the rapid absorption and removal of the enzyme, which normally is present in the intestinal content in low concentration.

Picon and Ramond (1899) found that extracts of *Taenia interme* fluid in culture media inhibited the growth of many common bacteria. Only the colon organisms resisted this action, although the growth was retarded.

Léon Frédéricq (1878) observed that certain ascarids of the dog (*Ascaris marginata*) could survive for several days *in vitro* in a pancreatic solution without alteration. Fermi and Parnossi (1895) made the observation that blood serum and living tissues possessed the same characteristics.

Weinland (1903) observed that many of the intestinal parasites, including varieties of *Taenia* and *Ascaris suilla*, exhibited antitryptic properties.

Dastre and Stassano (1903) observed that cestodes and nematodes escaped the digestive effect of pancreatic juices. They cited the earlier observations of various writers who stated that the "protoplasma vivant" possessed properties of resisting the proteolytic enzymes to a greater degree than did dead tissues. However, they state that extracts of *T. serrata* from the dog act against enterokinase rather than against trypsinogen. Hamill (1906) also made a study of this point, using intestinal worms.

Northrop (1939) found that for the earthworm (*L. terrestris*) and the meal worm (*Tenebrio militor*) the dead worms exhibited about four times as much tryptic digestion in 24 hours at 20° C. as did the living worms. The rate of digestion was determined by the increase in formol titration.

METHODS

The ascarids were collected from adult chickens sent to this laboratory for examination. However, samples were tested only from birds which appeared normal in other respects. The worms were washed free of mucus and wiped dry on a towel. They were then weighed and placed in a clean mortar. A small amount of washed quartz sand and 20 ml. of physiological salt solution (pH 7.2) were added for each gram of worm. The mass was then ground to a very fine pulp. This was placed in a tube and centrifuged at 3,000 r.p.m. for about 5 minutes. The supernatant fluid was treated as a 1:20 extract of the worm tissue.

Earthworms about 3–4 inches in length were collected from a wet sandy loam, washed free of soil, and placed in a jar with moist cotton for 24 hours at room temperature (23°–25° C.). They were then wiped dry on a towel. Since some of the worms still gave evidence of having soil in the intestinal tract, about 1 inch of the tail part was clipped off, and the soil was squeezed out of the remainder of the body. This left that portion apparently free of gross soil contamination. These portions were weighed and placed in a mortar with clean quartz sand and 20 ml. of physiological salt solution for each gram of worm material. This mass was ground as fine as possible, centrifuged, and the supernatant fluid considered as a 1:20 worm extract.

To determine the antitryptic properties of these extracts, the method of Fuld and Goss (1907) was followed, as cited by Kolmer. It is briefly as follows:

Solution of trypsin.—This solution was made by dissolving 0.5 gm. Bactotrypsin in 50 ml. of physiological salt solution, adding 0.5 ml. of normal sodium hydroxide solution, and making up to 500 ml. with saline. This was made fresh each time needed.

Casein solution.—One gm. of Bacto isoelectric casein was dissolved in 100 ml. decinormal sodium hydroxide solution with the aid of gentle heat. The solution was adjusted to pH 8.5 with N/10 hydrochloric acid, diluted with physiological salt solution to 500 ml., filtered, and placed in the refrigerator. This solution also was made fresh each time needed.

Acetic acid solution.—To 5.0 ml. of glacial acetic acid (c.p.) add 45 ml. of 50 per cent alcohol.

The technique of the test is indicated in Table 1.

The final pH of these mixtures was between 8.0 and 9.0, and, according to Northrop (1939), casein is digested most effectively at this pH range.

The tests were placed at 50° C. for 30 minutes, and the undigested casein was precipitated by adding 3 drops of the acetic acid solution to each tube. When the digestion was complete, the tubes remained clear. If a casein residue remained, a milky precipitate immediately developed.

TABLE 1

PROTOCOL FOR TITRATING ANTITRYPTIC ACTION

Solution (ml.)	Tubes											
	1	2	3	4	5	6	7	8	9	10	11	12
Trypsin*	0.0	0.01	0.05	0.1	0.2	0.4	0.6	0.8	1.0	1.2	1.5	2.0
Saline	2.0	1.99	1.95	1.9	1.8	1.6	1.4	1.2	1.0	0.8	0.5	0.0
Tissue extract	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Casein	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

* Stock solution diluted 1:4. This is determined by preliminary titration.

TABLE 2

ANTITRYPTIC ACTION OF WORM EXTRACTS

	Tubes											
	1	2	3	4	5	6	7	8	9	10	11	12
Trypsin solution	0.0	0.01	0.05	0.1	0.2	0.4	0.6	0.8	1.0	1.2	1.5	2.0
Trypsin alone	4*	4	1	.....	.....	.....	.....	.....	.....	.....	.....	.....
Ascaridia extract	4	4	4	4	4	4	4	4	4	4	2	1
Earthworm extract	4	4	4	4	4	4	3	1	.....	.....	.....	.....

* The figure "4" indicates no digestion of casein.

RESULTS

When the tube was clear, it was given a zero rating, indicating complete digestion. A 4+ indicated no digestion. The results of several typical titrations are shown in Table 2. It will be noted that the extracts from the *Ascaridia* are considerably higher in antitryptic action than those of the earthworm.

Calculated on the basis of the dilution of trypsin used, the Ascaridial tissues were about twenty times as active in inhibiting antitryptic action as were the earthworm tissues. Since this was a consistent finding, it appears significant.

CONCLUSIONS

From the observations made during these experiments, the following conclusions are drawn:

1. Both *Ascaridia* from the small intestine of the chicken and the common earthworm from the soil normally contain a certain amount of antitrypsin in their tissues.

2. The amount of antitryptic substance is appreciably higher in the extracts from *Ascaridia* than from the earthworm.

3. This antitryptic substance probably exerts an influence in protecting the living tissues from the action of endogenous proteolytic enzymes.

4. The reason for the differences exhibited by these extracts is unknown, but it may be suggested that under normal conditions of life the *Ascaridia* are constantly in contact with a tryptic enzyme, while the earthworms are not. However, the earthworm tissues are continually subject to the action of the worm's own tissue enzymes and of those from the rich flora of proteolytic bacteria in the soil.

5. Evidently such antitryptic compounds are widespread in living organisms in nature.

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